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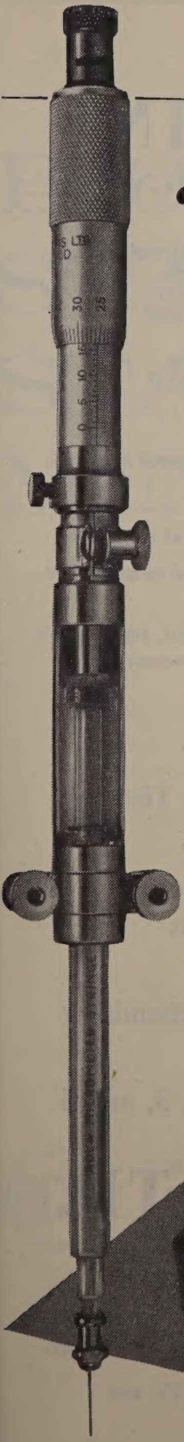

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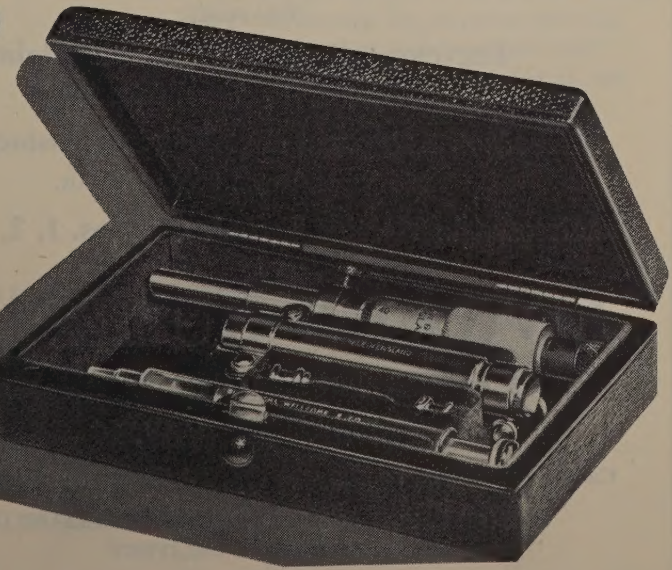

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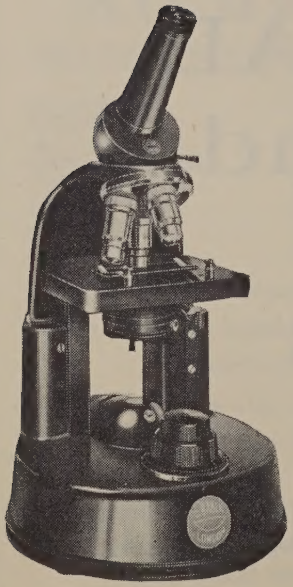

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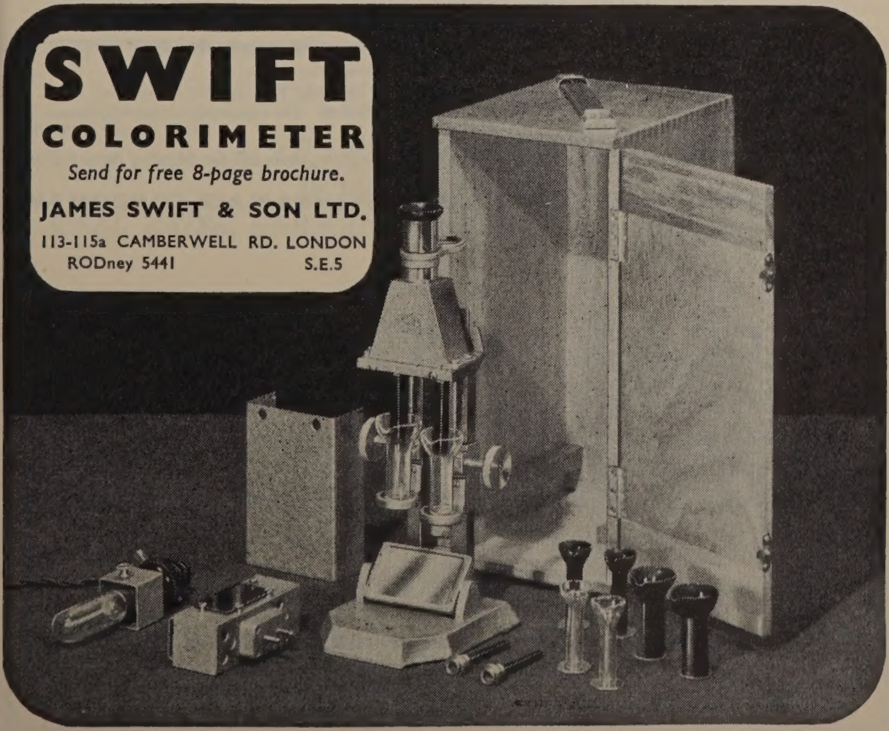

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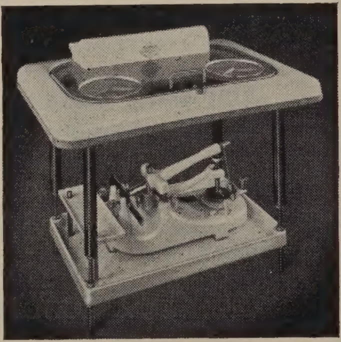

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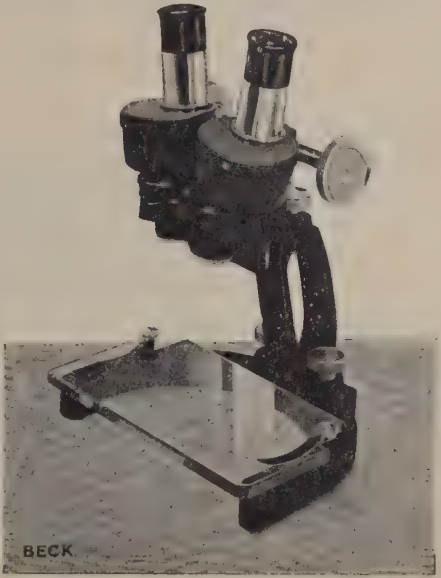

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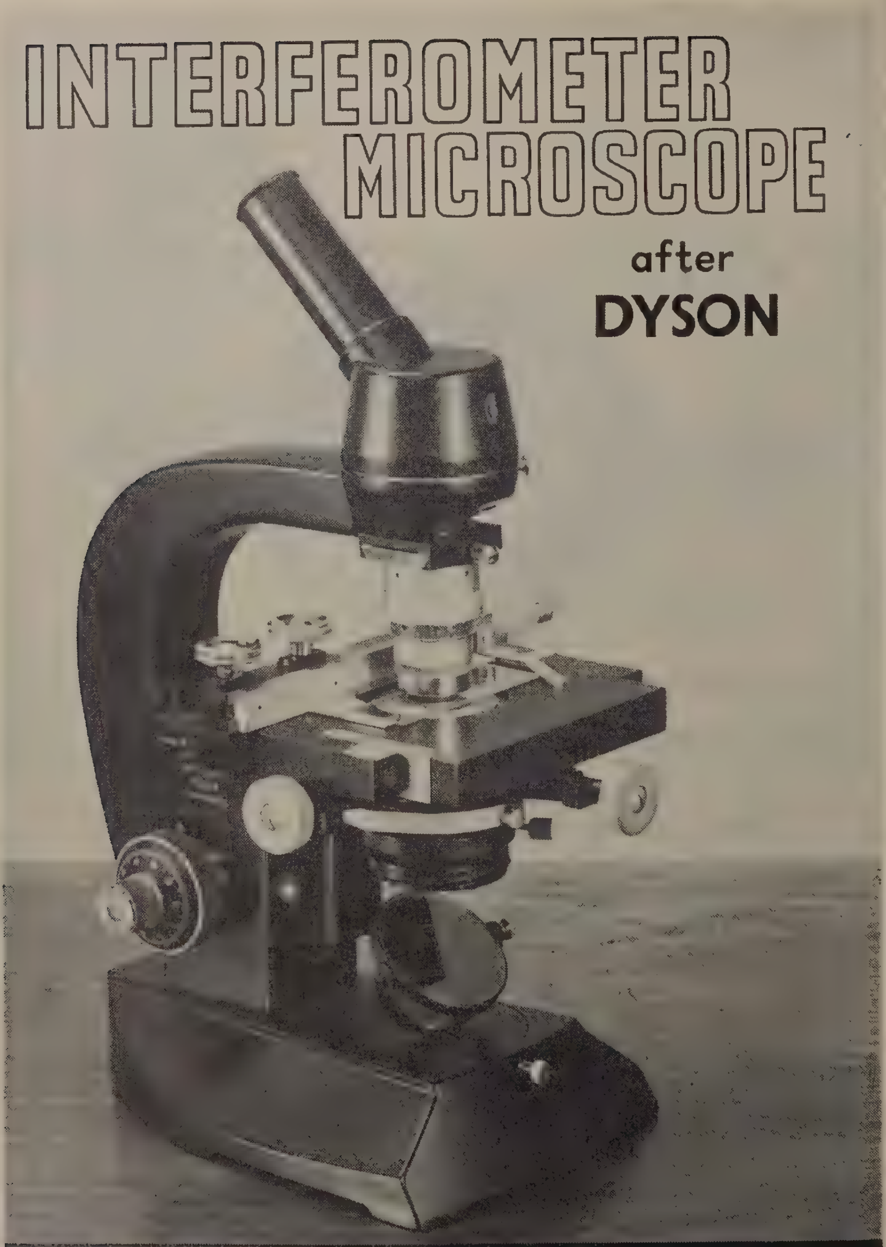

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Some Aspects of the Cytology of *Polycelis nigra*

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SUMMARY

The triclad, *Polycelis nigra*, has been found to be fully cellular. Gland-cells, undifferentiated cells, and the cell-bodies of muscle-cells, make up the parenchyma. The fine structure of the component cells of the parenchyma, nervous, and excretory systems, testis, pharynx, and epidermis is described. Acidophil secretion granules, produced by certain parenchymatous gland-cells, have a characteristic, doubly-banded ultrastructure which is not invariably associated with the property of adhesiveness. The parenchymatous cell-body of the muscles is often up to 10 μ from the muscle-fibre, to which it is joined by tenuous cytoplasmic connexions. The muscle-fibre itself consists of coarse and fine sets of hexagonally arranged myofilaments, but is unbanded. The basement membrane of the epidermis is composed of fine, banded fibrils, apparently randomly arranged in the plane of the membrane. Permeating the epidermis at a level just above the basement membrane is a system of extracellular spaces, which may have a hydrostatic function and assist in the extrusion of secretion granules. Epidermal sense organs, whose fine structure resembles the basal body of the cilia, are considered to have a functionally significant distribution on the surface of the animal. The rhabdites have been shown to develop in special cells of the parenchyma. Such rhabdite-forming cells, together with their contained rhabdites, have been found apparently passing through the basement membrane of the epidermis. As all the epidermal epithelial cells contain rhabdites, it is suggested that the epidermis as a whole is renewed by centrifugal migration of rhabdite-forming cells. The rhabdites themselves appear to consist of arginine and some tyrosine, together with a purine, probably adenine. They may be an excretory product.

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INTRODUCTION

MANY features of planarian cytology are still matters of controversy, from the question of the degree of cellularity in an animal widely regarded as being in part a syncytium, to that of the function of rhabdites, concerning which Prenant (1919) listed and rejected no less than 9 theories. Though triclads have been much used for work on regeneration, the cytology of regeneration in these animals has received little attention. In this study, electron microscopy and histochemical methods have been used in an attempt to resolve controversy and to provide a cytological description of the normal animal, in the light of which the special cytology of regeneration may be studied.

MATERIAL AND METHODS

Electron microscopy. Small animals 3 mm long were fixed in a 2·5% osmium tetroxide solution, buffered at pH 7·5, for 15 min, or in a 1% solution for 40 min, at 2° C, with veronal/acetate or *s*-collidine as a buffer. After fixation they were dehydrated rapidly in alcohol at 2° C and allowed to warm up to room temperature in propylene oxide. Two changes of this, each of 30 min duration, were used, and the animals were then transferred to a mixture of equal parts of propylene oxide and araldite for 3 h.

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At the end of 3 h the specimens were transferred to the pure araldite which was then polymerized, first at 40° C for 12 h, and then at 60° C for 56 h. Sections were cut on a Hodge thermal-advance microtome.

Animals were sometimes stained in bulk immediately after fixation for 36 h with 1% phosphotungstic acid, buffered with veronal/acetate at pH 5·0. Others were stained with a 1% aqueous solution of either uranyl acetate or uranyl nitrate. At other times, sections were stained for 2 h on carbon-coated, collodion-covered grids, with a saturated solution of uranyl acetate in 70% alcohol.

Specimens fixed with permanganate were prepared by the method of Luft (1956) and embedded in araldite.

The sections were examined with a Siemens Elmiskop 1 electron microscope.

Isolation of basement membrane. Animals were cut up into small pieces and left in a 1% solution of potassium phosphotungstate for 5 h at pH 7·0. The cells in contact with the membrane were not immediately killed, but they rounded up and could easily be stroked off the membrane with a glass needle. The cells had to be kept alive as long as possible in order that they might have time to round off. It might be thought that a balanced salt solution would be less toxic than a solution containing only one salt, but the sodium and calcium salts of phosphotungstic acid are more toxic than the potassium salt. Although the addition of 1% calcium hydroxide to the potassium phosphotungstate slightly decreases the toxicity, any addition of sodium rapidly kills the cells.

Histochemistry. Except where specified, all histochemical observation was made on frozen-dried material that had been vacuum-embedded in paraffin wax, sectioned at 5 μ, dewaxed, and stored in absolute alcohol for from 3 to 25 h to render it insoluble.

Even though a fixative has not been generally used for histochemistry, this does not emancipate the results from possible modification by the method of preparation of the tissues. Freezing-drying effects, although less is known about them than about fixation effects, cannot be ignored. It is unlikely that solution of proteins is occurring during or after prolonged treatment with absolute alcohol, but the lack of denaturation may have affected the tests for sulphydryl groups.

Observations on metachromasy were made on animals fixed in neutral formaldehyde / saline. Frozen sections of these were cut at 5 μ by attaching the animal directly to the chuck of a freezing microtome without using any embedding or supporting material, since this would interfere with the metachromatic effects. Sections were stained in 2×10^{-5} M toluidine blue for 24 h, with buffer at minimal ionic strength.

The periodic acid / Schiff (PAS) test was performed on formalin-fixed and on frozen-dried material. Some of the latter had been rendered insoluble by treatment with absolute alcohol. The rest was prepared with acetone, which is less polar, in order to have some check on the possible solubility of substances in alcohol. No differences between the two sets were observed. Control sections were tested for plasmalogens; others were deprived of lipids by means of xylene followed by a mixture of acetone and chloroform at 60° C.

Methyl green / pyronin staining was carried out on material embedded in wax of low melting-point. Both the methyl green and the pyronin solutions were extracted with chloroform until no more dye would dissolve in the chloroform.

All other histochemical methods were performed as described by Pearse (1953).

Collagenase digestion. Frozen-dried sections were treated for 26 h at 37° C with a solution of 30 mg of crude *Clostridium welchii* collagenase, with an activity of 22 Q units/mg and 52 K units/mg in 100 ml of buffer at pH 6·8, to which had been added an antiserum with anti-lecithinase, anti-hyaluronidase, anti-λprotease and slight anti-collagenase activity, in order to produce serologically specific collagenase. Evidence of the solution of collagen was obtained with polarized light and by staining with aniline blue.

Extraction of rhabdites. Frozen-dried animals were homogenized in a Potter glass homogenizer and centrifuged at 2° C for 5 h at 2,000 $\times g$ in a density gradient of benzene and carbon tetrachloride. Rhabdites formed the bulk of the top layer. They were purified by isopycnic centrifugation with benzene and chloroform. Paper chromatography was carried out with 65:35 *iso*-propanol / 2N HCl overnight. Chromatogram spots were detected by photographing in ultra-violet light.

Light microscopy. For routine microscopic examination, specimens were stained by the osmium / ethyl gallate procedure of Wigglesworth (1957). This was combined with double embedding in agar and ester wax. Sections were cut at 0·8 μ and 0·4 μ with a standard Cambridge rocking microtome. For examining individual cells, animals were macerated by the osmic-acetic method of the Hertwigs (1879). Specimens were left in the fixative for a maximum of 5 min and were then macerated in 0·02% acetic acid for 2 days. The macerated material was teased and stained in picro-carmine.

Results

Parenchyma

The parenchyma of planarians is almost always supposed to be non-cellular, and even Hyman (1951) refers to it as a syncytium, with associated free cells. This view was originally put forward by Hallez (1879) and considerably elaborated by Prenant (1922). With the electron microscope, however, the parenchyma can be resolved into its component cells. It is seen to be made up of the cell-bodies of muscle-cells, of gland-cells (including the rhabdite-forming cells), and rounded cells that appear to be undifferentiated.

The gland-cells may be divided into 3 groups on the basis of the staining characteristics and morphology of their granular secretions.

Cyanophil cells. The secretions of these stain strongly with alcian blue, are PAS-positive, and show β-metachromasy at pH 6·4. Owing to their unicellular nature, their apparently random distribution in the ventral region of the animal, and their wide variety of shapes, it has not been possible to verify that the unicellular glands staining positively with one technique are of the same type as those staining with another. It is possible, therefore, that this group of cyanophil cells includes more than one histochemically-distinct type. Under the electron microscope, however, in material stained with phosphotungstic acid, only one morphological type of pale granular secretion has been found. This consists of globules, 0·5 μ to 0·8 μ in diameter, which, unlike those of the cyanophil secretion of the pharynx, are uniformly pale. The secretion is discharged into structures called gland-cell 'necks' by Hyman (1951), but which, in fact, contain no gland-cell cytoplasm but are membranous tubes lined with longitudinal, non-striated fibres, 200 to 220 Å in diameter. The tubes penetrate through the parenchyma and basement membrane, and open to the exterior through the centre of the epidermal cells.

Acidophil cells. The granules of these cells vary in shape from spheres 0·5 μ in diameter, to elongate bodies 1·5 μ long which appear almost oblong in longitudinal section. Each is invested with a double membrane. In the living animal these highly refractile granules can be seen to circulate through the parenchyma, moving through tubules which are often only wide enough for granules to pass along singly. In other places the granules collect in what are, apparently, reservoirs. This secretion, which is usually called an adhesive substance, is discharged to the exterior through the epidermal cells. Discharge-sites occur on the dorsal surface but are particularly abundant around the lateral margins of the animal. No adhesive papillae have been observed in well-fixed preparations, but the 'papillae' produced in poorly fixed animals are formed from coalesced masses of these acidophil granules.

The granules are acidophil even at high pH levels, a feature on which the Alfert and Geschwind (1953) test for histones is based; this diagnosis of histones is confirmed by a strong positive result with the Sakaguchi test for arginine. They also give a faint positive reaction with Millon's reagent, when the modification of Bensley and Gersh (1933) is used. With the PAS technique the granules themselves give a negative result, but the fluid in which they are suspended is often positive, though it does not stain with toluidine blue at pH 6·4. These results are in substantial agreement with those of Pedersen (1959*a*).

Pedersen found a strong positive reaction for both sulphydryl groups and disulphide groups by the technique of Barrnett and Seligman (1952, 1954). A positive result was obtained in *Polycelis* with the ferricyanide method of Chèvremont and Frederic only after reduction with sodium thioglycollate at pH 8. This indicates that disulphide groups are present, and the apparent absence of sulphydryl groups might be due to insufficient denaturation of the protein by the freezing-drying technique. This suggestion is a corollary of the finding by Anson (1940) that native egg albumin does not reduce ferricyanide even at pH 9·6, although sulphydryl groups and tyrosine are present. Anson showed that sulphydryl groups could be demonstrated by the reduction of ferricyanide only in denatured egg albumin.

Under the low-power of the electron microscope, the granules exhibit a coarsely banded ultrastructure, similar to that of the acidophil granules of *Planaria vitta* described by Pedersen (1959*a*). Throughout each granule, bands are arranged transversely to its long axis; the dark bands are narrower than the pale bands, and the distance from the centre of one dark band to the centre of the next is commonly 420 Å in those sections of the granules which appear to be accurately longitudinal. Other granules, however, display narrow dark bands with centres only 315 Å apart, and, with high resolution, these granules also show commonly two (fig. 1, C) (or less commonly, one (fig. 1, B)) yet finer bands parallel with, and equally spaced between, the narrow, dark, transverse bands. In addition to these transverse bandings, a further fine but intensely staining banding is present, apparently at right angles to that previously described, and parallel with the long axis of the granule (fig. 2, A). Although the coarse transverse and the longitudinal striations appear to be lamellar, the fine transverse bands which are often double but occasionally single, suggest a transverse hexagonal array of molecules between the lamellar transverse bands.

The interband distance of the longitudinal striations is usually 180 Å as measured in accurately transverse sections of the granules. A granule was judged to have been accurately sectioned transversely when it had an exactly circular outline with the double membrane visible all round, and when the transverse banding was absent. It was found that the transverse bands were sufficiently dense for them to be visible even when sectioned so nearly longitudinally that a single band covered seven-eighths of the granule area.

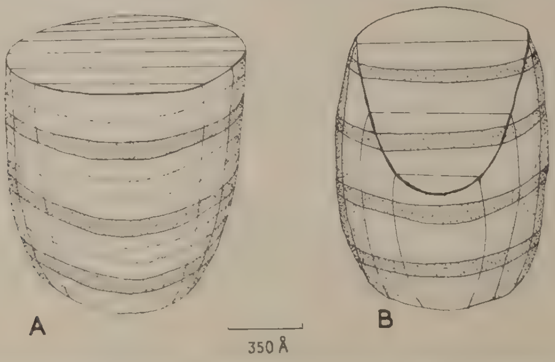

Fig. 2. Diagrams of banded acidophil granules of the body region, showing the coarse transverse and longitudinal bandings. A, granule showing two fine transverse bands between the coarse dark bands. B, granule showing the plane of the section which gives the effect of the longitudinal bands being parallel to the transverse bands.

In oblique sections of the granules, the longitudinal and transverse banding systems should appear at varying angles to one another depending on the degree of obliquity of the section. In certain cases it is possible for both longitudinal and transverse bandings to appear more or less transverse (figs. 1, A; 2, B). Under such circumstances, the bands should be considerably more widely spaced than the minimum interband distance. In some sections however, in which the longitudinal bands appear nearly transverse, their spacing is as low as 70 Å. It is therefore considered likely that the spacing of the longitudinal bands varies from granule to granule, although whether this is correlated with a change in shape of the granules cannot be decided until a method is evolved for cutting oriented sections of the granules.

Fig. 1 (plate). Preparations fixed with osmium, stained with uranyl acetate. All at same magnification. Sections of banded acidophil granules.

A, very fine longitudinal striations almost parallel to the coarse transverse bands.

B, one fine transverse band (indicated) between two coarser transverse bands.

C, coarse transverse bands with two finer transverse bands between each pair.

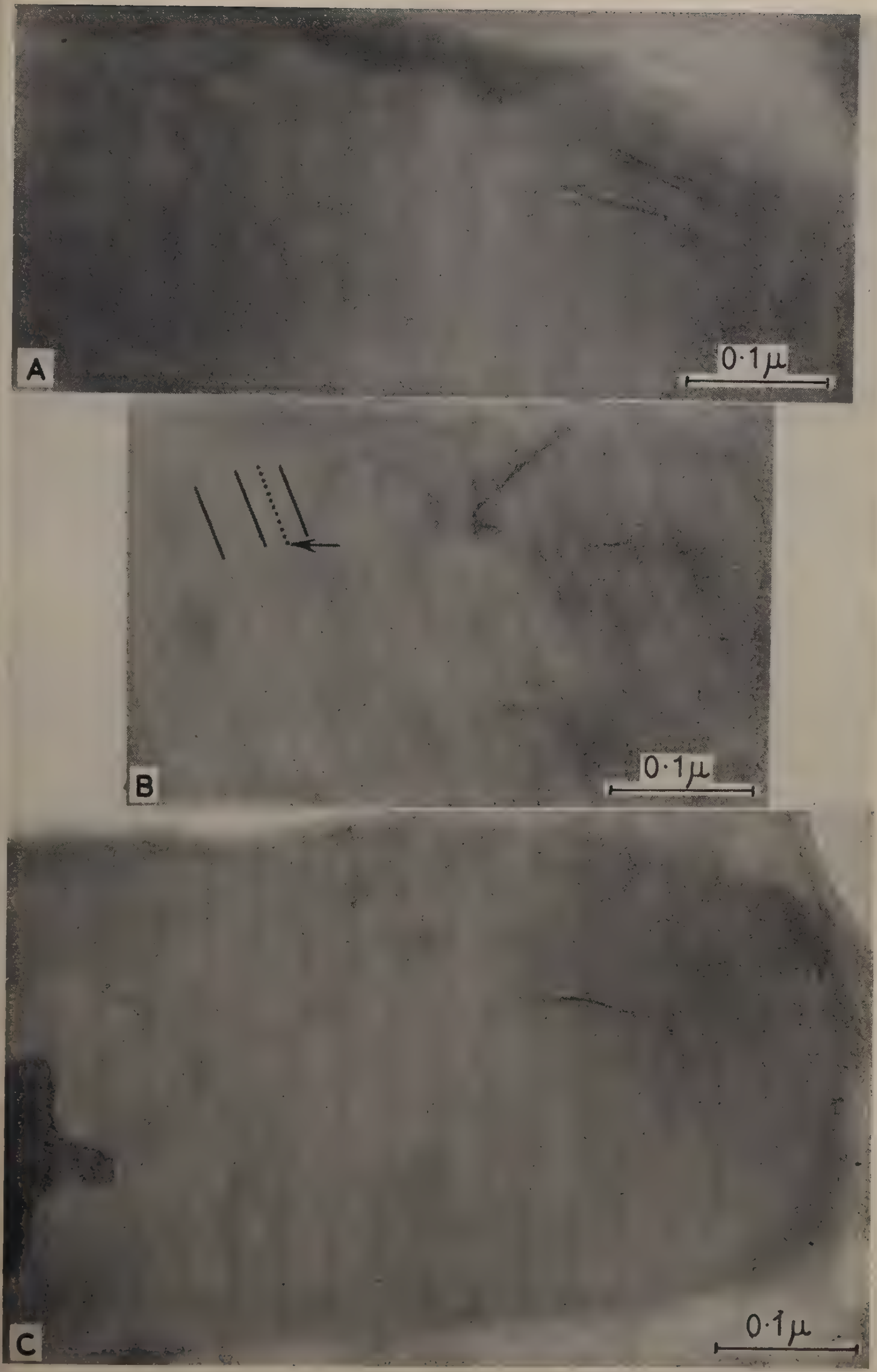

Fig. 1

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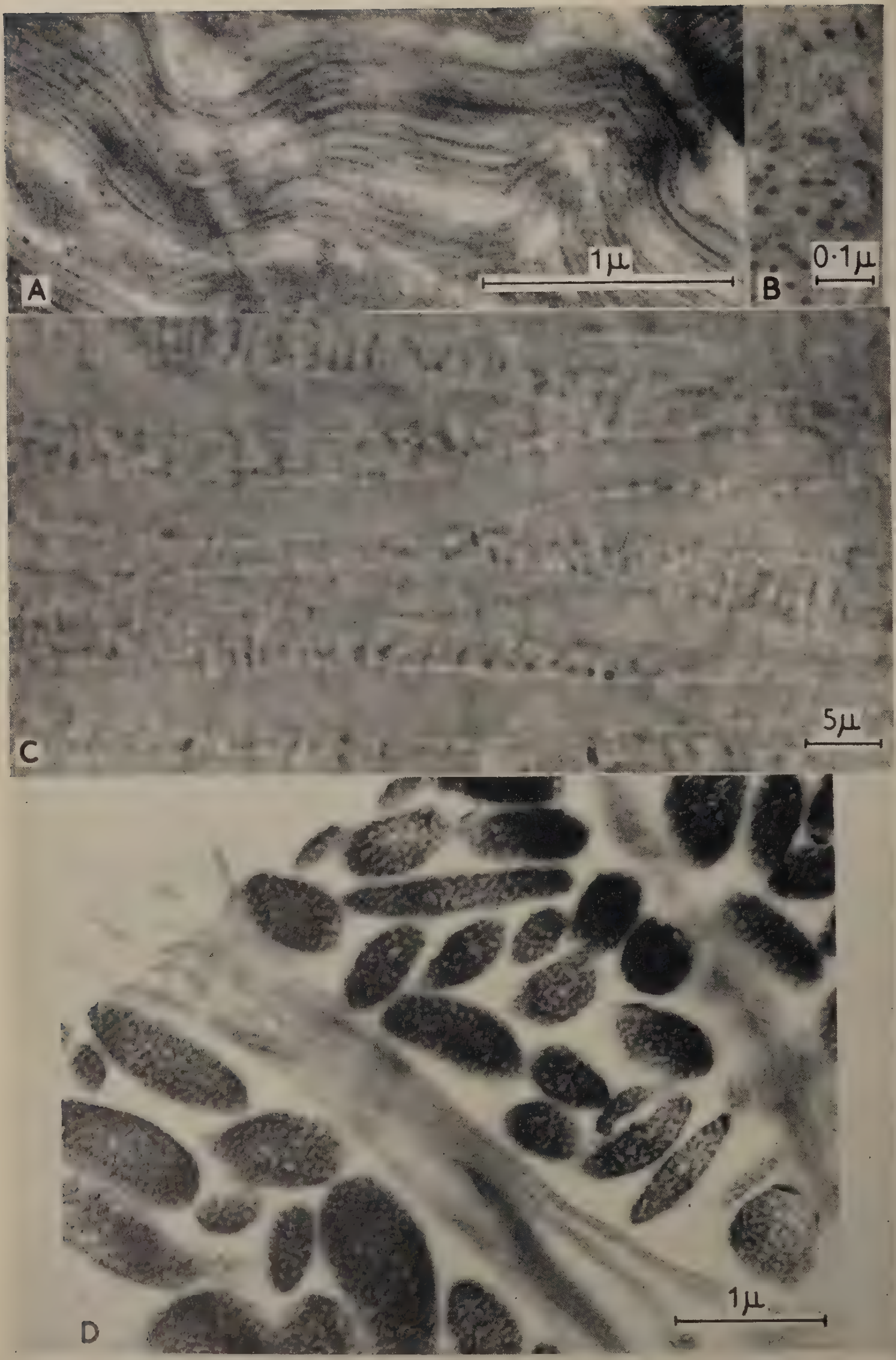

Fig. 3

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The size and shape of the granules cannot be correlated with their situation in the body of the animal, for though most of the granules near the epidermal surface are about 1·5 μ long and 0·8 μ in diameter, granules of this size and shape are sometimes visible in sections of the cytoplasm of the formative cell. In this situation, the granules are associated with a well-developed endoplasmic reticulum and numerous mitochondria.

In the membranous tubes, the granules released from the formative cytoplasm are suspended in a fluid which also contains small dense granules about 120 Å in diameter. In the lower region of the epidermis, the tube may itself be surrounded by a clear, fluid-filled space into which open large vesicles from the neighbouring cytoplasm. This system of epidermal spaces investing the tubule may be associated with the discharge of the granules, and is probably a part of a system of fluid-filled spaces to be described later in connexion with the epidermis.

Fusion of the granules before discharge, as found by Pedersen (1959*a*) in some of his preparations, has not been seen in well-fixed animals. Individual granules have been observed in stained trails of the animals, where they form very elongate threads. In living animals treated with acetic acid, such threads were found in the electron microscope to be faintly banded with very wide pale bands and less wide, slightly oblique, dark bands. Elongation in this case is most likely to have been caused by stretching of the granule, however, rather than by internal reorientation of molecules.

In order to find whether adhesiveness is always associated with such a banded structure, reminiscent of discharged trichocysts, the musculo-glandular adhesive organ of the flatworm *Bdellocephala* was sectioned. The granules produced in this organ are just over 1 μ in diameter and strongly acidophil. Their ultrastructure, however, is not banded but coarsely reticular (fig. 3, D).

Rhabdite-forming cells. The cytoplasm of these cells stains orthochromatically with toluidine blue at pH 3·6 when a 10^{-5} M solution is used. Similar staining is displayed by the cytoplasm of the gland-cells and of the vitelline cells. Electron microscopy reveals that all these cells have a well-developed endoplasmic reticulum. Each rhabdite-forming cell contains several rhabdites in what are inferred to be different stages of development. At the earliest stage that has been found, the rhabdite is a pale, cigar-shaped body 5 μ long and 0·8 μ broad, surrounded by a double membrane. Mitochondria, endoplasmic reticulum, and piles of flattened vesicles are grouped closely round it and it is

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FIG. 3 (plate). A, preparation fixed with osmium, stained with P.T.A. Longitudinal section of circular muscle showing two types of myofilaments. The structure on the right is an acidophil granule.

B, preparation fixed with osmium, stained with P.T.A. Transverse section of circular muscle, showing coarse and fine myofilaments in hexagonal arrays.

C, macerated preparation. Outer longitudinal muscle layer of pharynx, showing lateral cytoplasmic processes of myoblasts containing mitochondria.

D, preparation fixed with osmium, stained with P.T.A. Longitudinal section of musculoglandular adhesive organ of *Bdellocephala*, showing reticulate acidophil granules.

not possible to say which, if any, of these components is most concerned in its formation. The internal structure, which develops at a slightly later stage, consists of fibrils 120 Å wide spaced 300 Å apart. These are oriented longitudinally in the rhabdite and extend from one end of it to the other (fig. 4, A). The size-changes of rhabdites during their formation have not been worked out but it is considered that size is not a reliable criterion for estimating the age of a rhabdite. At a later stage, granules which stain intensely with phosphotungstic acid and with uranyl acetate and are dense in material fixed in permanganate, become deposited on the fibrils (fig. 4, B). The granules increase in number and size until the original fibrous structure is completely obscured (fig. 4, C).

The granule size varies considerably in different fully developed rhabdites, and in some cases this may be ascribed to the fixation, for the large granules can sometimes be seen to consist of incompletely coalesced finer granules. The large granules are occasionally drawn out to form long threads within the rhabdite. Poorly fixed and what appear to be well-fixed rhabdites often occur within the same cell. The worst fixed part of a rhabdite, as judged by the lack of internal ordered structure and the variety of effects produced, is frequently that nearest to the cell surface. This phenomenon is particularly noticeable in epidermal rhabdites and may be due to convulsive movements of the animal.

The best-fixed rhabdites (fig. 5) show a double membrane enclosing a relatively palely staining cortex of fine granules, while the remainder of the rhabdite is filled with rather larger granules up to 100 Å in diameter. The use of *s*-collidine as a buffer, in place of veronal / acetate, increased the proportion of finely granular rhabdites present and decreased the average size of the individual granules.

Rhabdites embedded in methacrylate often show a surrounding clear space as reported by Pedersen, who considered that these spaces might be artifacts due to extraction of material during fixation or dehydration. This effect is not shown in specimens embedded in araldite and is regarded as a polymerization artifact due to the use of methacrylate.

Rounded, apparently undifferentiated cells have been observed and probably include the types called neoblasts by Pedersen (1959*b*) and parenchyma cells by Murray (1931). They are characterized by a high frequency of mitosis and Pedersen finds that the neoblasts have a strong cytoplasmic basiphilia.

FIG. 4 (plate). A, preparation fixed with permanganate, unstained. Section of rhabdite-forming cell with a developing rhabdite, showing fine longitudinal fibrils. The clear space at the bottom of the photograph is the nucleus.

B, preparation fixed with osmium, stained with P.T.A. Section of a rhabdite-forming cell, showing a later stage in rhabdite-formation. Granules have been deposited on the fibrils. At the bottom, a more fully developed rhabdite.

C, preparation fixed with osmium, stained with P.T.A. Transverse section of an epidermal cell, showing the palely staining granular sheath around each rhabdite. The dense granules in the centre of the right-hand rhabdite are arranged in longitudinal rows.

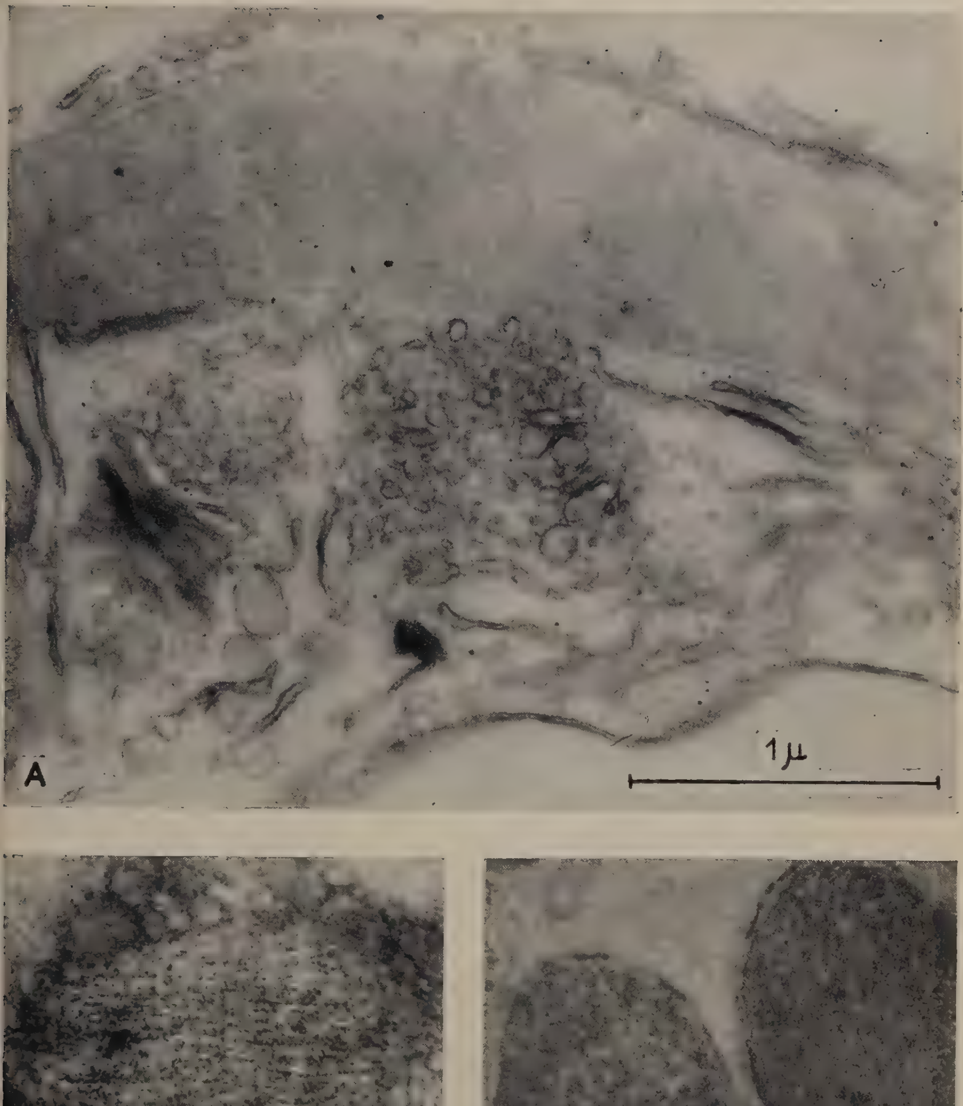

Fig. 4

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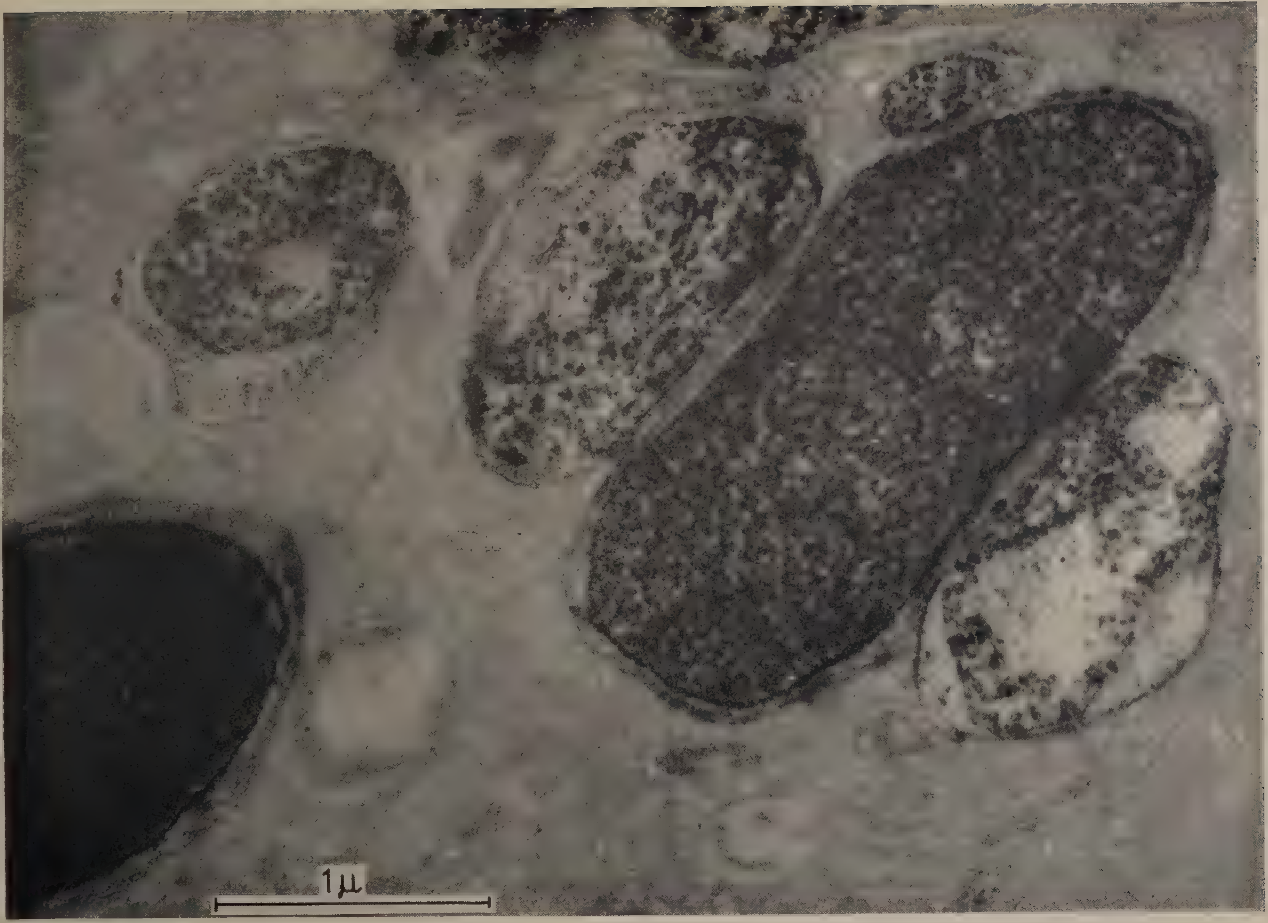

FIG. 5

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These characters alone, however, do not suffice to define and distinguish the cell types. A single cell of this undifferentiated kind was found to be binucleate under the electron microscope. The group undoubtedly includes transverse sections of the bases of gland-cells, the proximal cytoplasm of which may be free of secretion granules.

Individual muscle-cells were isolated from the body and the pharynx by the Hertwigs' maceration procedure. The muscle-cell appears as a contractile cylinder, up to 150 μ long in the body region but up to 250 μ in the pharynx, surrounded by a delicate protoplasmic sheath prolonged into processes as described by von Gelei (1927) (fig. 3, C). The latter arise, for the most part, on opposite sides of the sheath, and near the tips of the cell their bases lie on a spiral around it. The largest of the processes, forming a large proportion of the parenchyma, contains the nucleus. This may be up to 10 μ away from the contractile sheath. The smaller processes, which in material macerated by the Hertwigs' method appear as vesicles, and which von Gelei believed to have a nutritive function, are shown by the electron microscope to be aggregates of mitochondria. These aggregates are bounded by a plasma membrane and are connected to the contractile sheath by tenuous necks of cytoplasm. Preparations stained with osmium / ethyl gallate showed that myofilaments are absent from the centre of the contractile cylinder of the larger muscle-fibres. No muscles are striated, but coarse and fine myofilaments are present (fig. 3, A, B). Six fine myofilaments of diameter 70 Å are arranged around each myofilament of diameter 190 Å, and thus the myofilaments are distributed in two hexagonal arrays. The length of individual myofilaments could not be determined in sections, since the fixed cells are all to some extent buckled.

The parenchymal pigment of *Polycelis nigra* does not occur in special pigment cells, but as granules in the cytoplasm of the muscle-cells in particular and of some nerve-cells. It is supposed to be a melanin pigment since it is a brown pigment that reduces ferric ions to ferrous, in which state they can be detected by the simultaneous or subsequent use of ferricyanide. This implies an ability to complex with ferrous ions, which is also a characteristic of melanin. Moreover, the pigment is completely bleached by permanganate, but completely insoluble in organic solvents. It occurs in the form of spherical granules 0·4 μ in diameter. These have no visible internal structure, and banded melanin precursors, as reported in mammalian tissues by Rogers (1956) and others, have not been observed.

Nervous system

In material fixed with osmium, the nerve-tracts have been found to contain at least two types of nerve-cell. One has a very elongate nucleus surrounded by a small amount of cytoplasm; the other, a spherical nucleus and abundant, vacuolated cytoplasm with melanin granules; both types appear to be bipolar.

FIG. 5 (plate). Preparation fixed with osmium, stained with P.T.A. Fully developed rhabdites in an epidermal cell. A double membrane is visible in places around each rhabdite. The structure on the left is a mucus-tube.

Several other types which have not yet been characterized occur around the brain. Nervous elements have been identified under the electron microscope by their position in relation to other tissues in a survey micrograph, and by the tubular nature of the components, the range in diameter of which (0·2 μ to 2 μ) corresponds to that observed in the light microscope.

No myelination or Schwann cells are present (fig. 6, B), but each tubular component visible with the light microscope is surrounded by a double cell-membrane of the same thickness as that which surrounds the muscle-cells. Within each of the structures so delimited is a further system, apparently of double membranes or fibres approximately 200 Å in thickness, and thus of about the correct size for neurofibrils. Owing to the length of each double structure sectioned, this may be a system of membranes, or of fibres with a considerable degree of parallel orientation. They are spaced laterally at approximately 0·1 μ intervals but sometimes anastomose, although they do not appear to form completely enclosed tubes. It is considered probable, therefore, that the structures visible under the light microscope are axons rather than bundles of axons. The tips of some of the smaller axons are filled with granules and vesicles 430 Å in diameter.

Excretory system

A flame cell seen under the electron microscope in permanganate-fixed material consisted of up to 60 cilia, each with a ciliary membrane, fused together by amorphous material between them. Near the base of the flame, the fused cilia are interspersed with microvilli. This structure is precisely that found by Kümmel (1959) in the flame cells of *Fasciola*. There is no trace in *Polycelis*, however, of a ribbed membrane like that present in *Fasciola*. Numerous mitochrondria are present in the capillary surrounding the flame.

Reproductive system

The stages of spermateleosis have been examined in material fixed in buffered potassium permanganate. In the spermatid, small mitochondria are aggregated around the spherical nucleus. These mitochondria appear rounded, but this may be due to the swelling action of the fixative as reported by Luft (1956). A pile of flattened vesicles is situated at the proximal end of the spermatid, close to the nuclear membrane. A system of a few double membranes is present, parallel with, and immediately beneath the plasma membrane, but whether or not they are associated with cytoplasmic granules has not been determined. None are visible; but permanganate is known to dissolve ribonucleic acid. A single flagellum develops from the distal end of the cell,

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FIG. 6 (plate). Preparations fixed with osmium, stained with P.T.A.

A, transverse section of basement membrane, showing two mucus-channels. The epidermal cells are anchored to the basement membrane by large prolongations. Part of the system of epidermal intercellular spaces is also visible. The groups of darkly stained granules on the lower surface of the basement membrane are believed to be fat-droplets.

B, transverse section of dorsal region of the parenchyma, showing basement membrane, muscle-cells with pigment granules, and nerve-fibres.

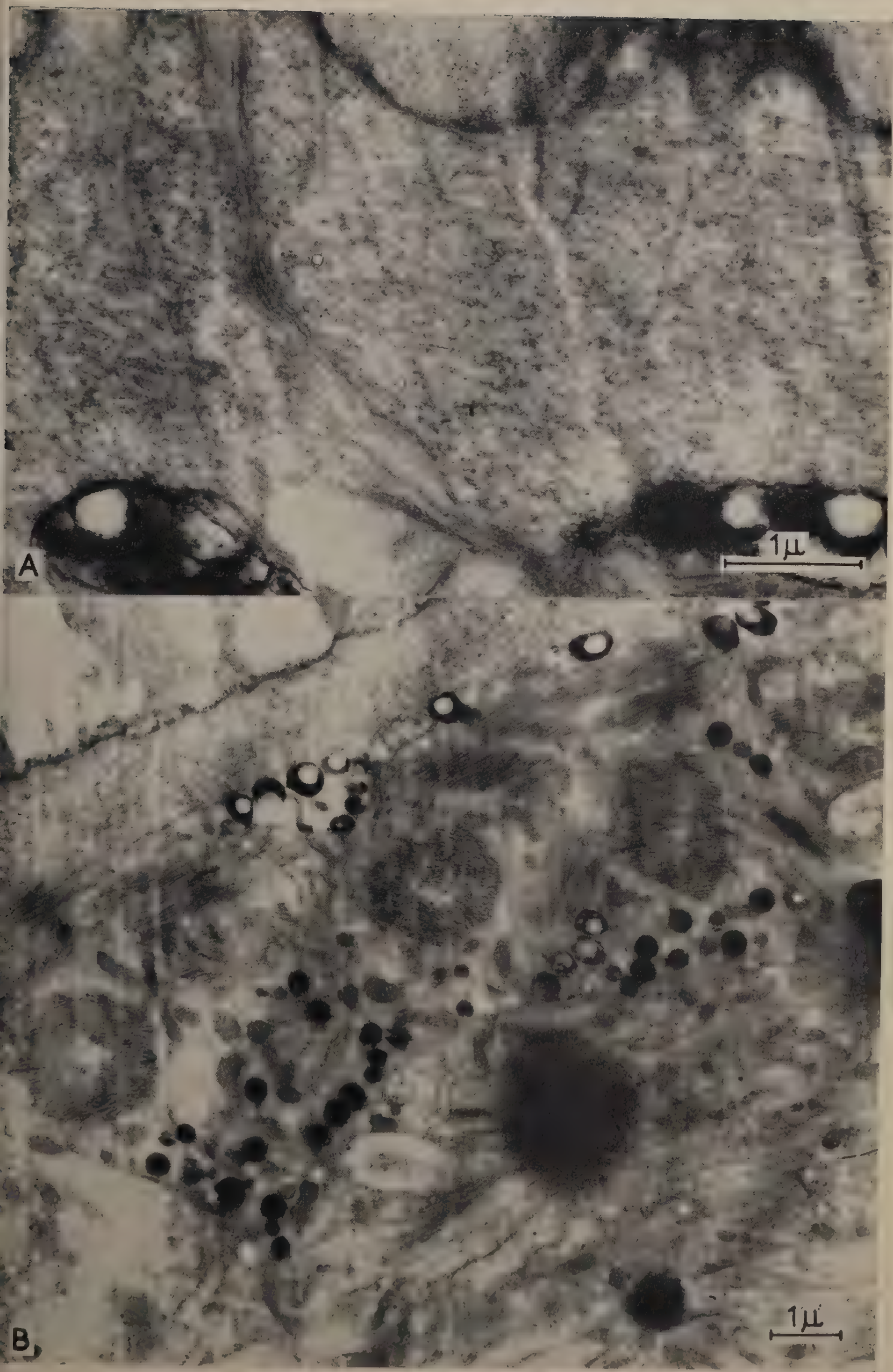

FIG. 6

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and the nucleus is elongated towards this end. At this stage, the contents of the nucleus appear darker and more granular than the contents of the spermatid nuclei. As the nucleus with its surrounding mitochondria becomes more elongated, some cytoplasm remains at the proximal end as a bleb, which is probably resorbed at a later stage. The nuclear contents now appear in transverse section as dark, convoluted membranes which in sagittal section may be seen as fine, parallel, dark threads arranged longitudinally. The nucleus eventually extends almost from tip to tip of the spermatozoon, one end of the nucleus being embedded in the pile of flattened vesicles which form the acrosome. The origin of the two flagella of the mature spermatozoon has not been studied.

The mature spermatozoon is 0·9 μ at its widest diameter, and at this level the nucleus has a diameter of 0·75 μ. Nearer the tips of the spermatozoon, where the overall diameter decreases, the ratio of the dimensions of the nucleus to those of the outer membrane remains approximately the same. Beneath the outer membrane are some 20 to 30 longitudinal fibrils each 120 Å in diameter, which continue into the pointed tip not occupied by the nucleus. The nucleus is surrounded by a thick double membrane, sometimes with a clear space beneath. The contents of the nucleus appear to consist of two types which occur in approximately equal numbers, with an apparently random distribution among spermatozoa which are otherwise identical. One type, in transverse section, has a dense reticular structure evenly distributed across the nucleus and appearing as threads parallel to the nuclear membrane in longitudinal section. The other type in transverse section appears as 6 dense areas arranged peripherally within the nuclear membrane and interspersed with pale, amorphous material. In longitudinal section, the nuclear contents of this type may be seen to consist of a longitudinal spiral structure with a pitch of 45°, formed from 6 dense wide rods which alternate with 6 much finer fibres. The spirally arranged dense material can be seen at an early stage of formation in spermatid nuclei which are just beginning to become elongated. It is not known whether these differences in nuclear contents are due to differences in the level of sectioning of mature sperms, or whether they represent age differences, or even if they indicate a true dimorphism of spermatozoa.

The rest of the reproductive system has not been specially investigated apart from the examination of osmium-fixed material to confirm that the system is fully cellular.

Digestive system

The pharynx has been investigated mainly by use of Wigglesworth's osmium / ethyl gallate method, the Hertwigs' maceration procedure, and electron microscopy.

The outer epidermis consists of ciliated cells, the nuclei of which are insunk approximately 25 μ below the surface (fig. 7, A) to form a 'zone of nuclei' beneath the outer longitudinal and circular muscles. The outer region of these cells is a layer of cytoplasm 2 to 3 μ thick, containing small round mitochondria 0·3 μ in diameter (fig. 7, D); the surfacecil ia are 3·5 μ long and interspersed with microvilli. The cytoplasm also contains elongate vesicles with clear contents; in some cases these appear to be connected to the surface and resemble pinocytosis vesicles. Attached by long necks of cytoplasm, up to 20 μ in length, to the lower surface of the outer cytoplasm are large vesicles, 2 μ by 4 μ (fig. 7, A), insunk sometimes to the level of the nucleus. Under the electron microscope, each vesicle can be seen to contain a few very large mitochondria, all of which are several μ long and loosely coiled (fig. 7, F, G). These vesicles form a layer between the insunk nuclei and the circular muscle, but the significance of the virtual isolation of these very large mitochondria from the rest of the cell is unknown.

Below the outer layer of cytoplasm occurs a cylinder of longitudinal muscles and beneath this a layer of circular muscles, all with their nuclei in approximately the same zone as the nuclei of the epidermal cells.

Between the longitudinal muscle and the lower surface of the outer cytoplasmic layer of the epidermal cells, is a layer of intercellular substance (fig. 7, D). The substance of this layer is finely granular, faintly positive with the periodic acid / Schiff test; it occurs between all the cells of the pharynx below the level of the cytoplasmic epidermal layer. It does not occur between cell membranes within this layer, and where it comes into contact with the lower surface of this cytoplasmic layer, it forms a membrane, 200 Å thick, which does not appear to be fibrous. The intercellular substance is strongly chromotropic when stained with 2×10^{-5} M toluidine blue, at pH 6·4, but is not stained at pH 3·6. At pH 6·4 it shows γ-metachromasy, being stained a very pale pink.

In the interior of the pharynx wall are two types of gland-cell, one producing a cyanophil and the other an acidophil granular secretion. The acidophil granules are more numerous than the cyanophil near the lumen of the pharynx; they are spherical, with an average diameter of 0·8 μ, and, apart from a surrounding membrane, appear uniformly dense under the electron microscope (fig. 7, E). The cyanophil granules give a positive result with the periodic

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FIG. 7 (plate). A, macerated preparation. Cell from outer insunk epithelium of pharynx. The spaces below the ciliated outer cytoplasm are normally occupied by longitudinal muscle-cells. The arrow indicates a vesicle, containing mitochondria, attached to the outer layer of cytoplasm by a long narrow neck.

B, preparation fixed with osmium, stained with P.T.A. Fully hydrated basiphil mucus from inner region of pharynx wall.

C, preparation fixed with osmium, stained with P.T.A. Two slightly hydrated groups of basiphil mucus from inner region of pharynx wall. They are surrounded by intercellular substance.

D, preparation fixed with permanganate, unstained. Section of ciliated outer layer of pharynx showing small mitochondria, ciliary rootlets, and partially hydrated basiphil mucus. Below the cytoplasmic layer is intercellular substance and part of a muscle-cell.

E, preparation fixed with permanganate, unstained. Acidophil granules of pharynx.

F, preparation fixed with osmium, stained with P.T.A. Section of vesicle from cell of outer insunk epithelium of pharynx, showing the large mitochondria with stained contents.

G, preparation fixed with permanganate, unstained. Section of a vesicle from a similar situation to F. Mitochondrial membranes are stained intensely, but contents are unstained.

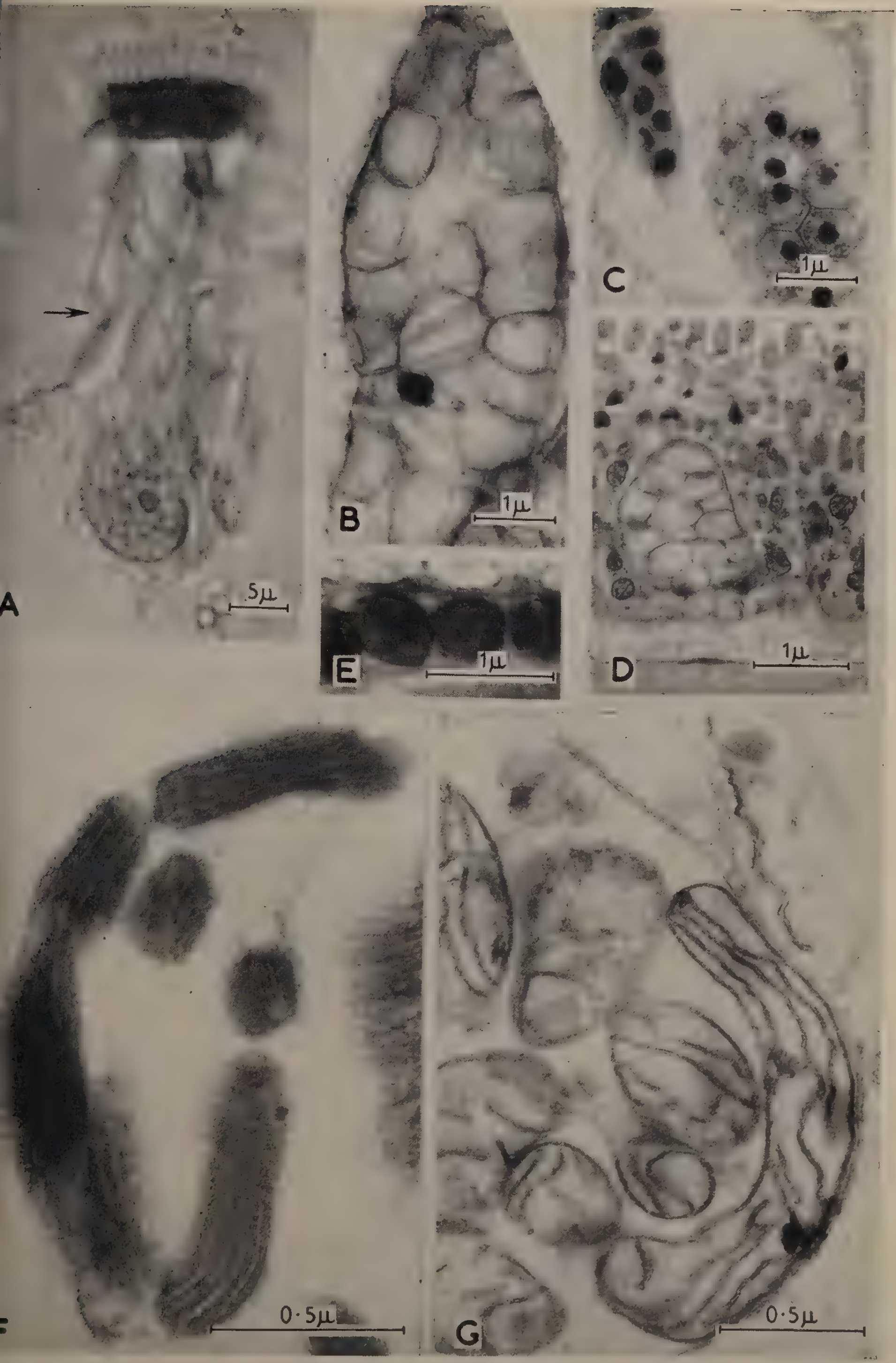

FIG. 7

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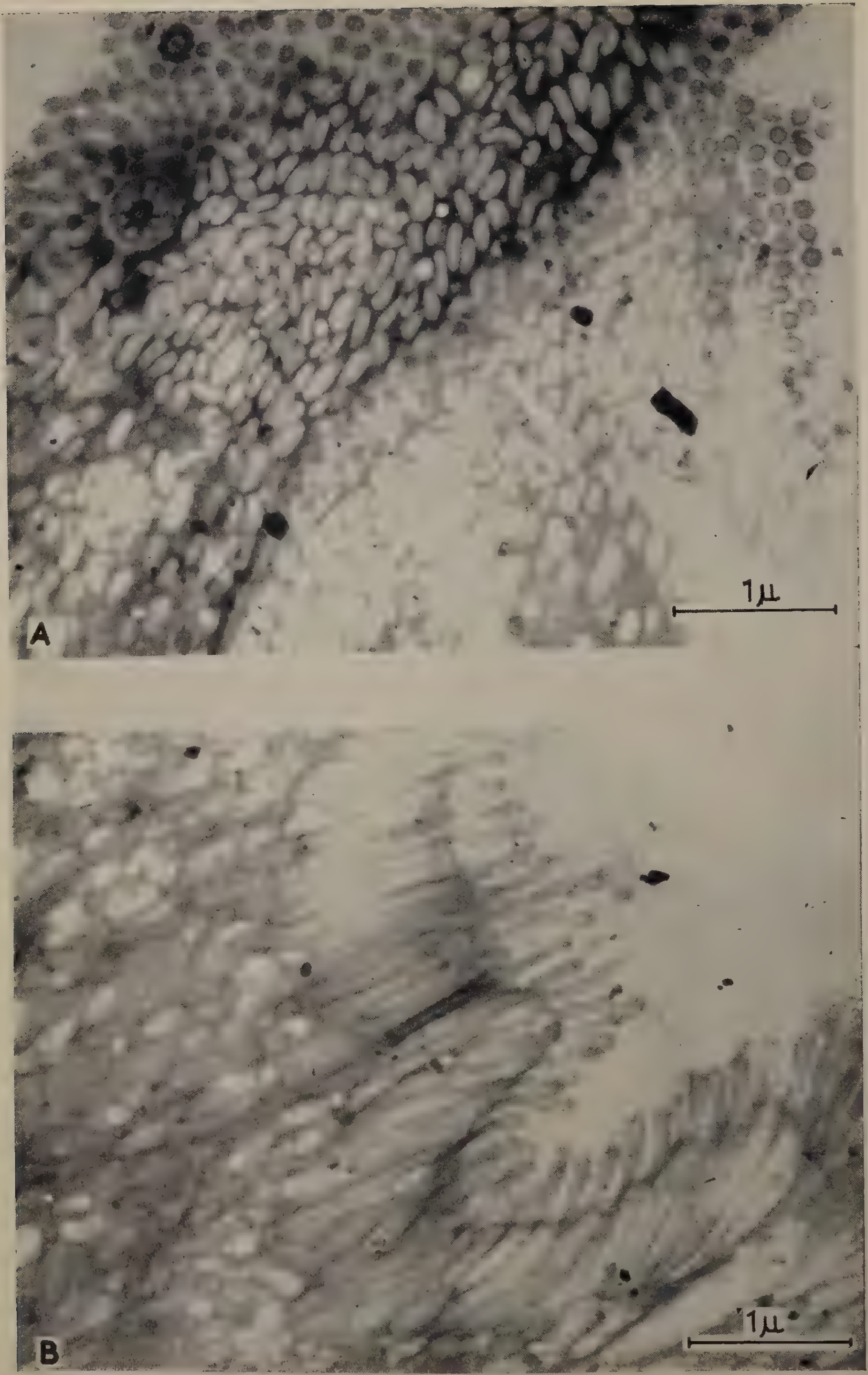

FIG. 8

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acid / Schiff test and show β-metachromasy with toluidine blue at pH 6·4. They appear to be a mucoprotein. When first secreted, these mucoprotein granules are rather dense with a single very dense region in each (fig. 7, C). Farther away from the originating cell, the granules become considerably less dense (fig. 7, B), but even when passing through the outer cytoplasmic layer of the epidermis, many still retain the very dense region (fig. 7, D). In this respect the mucus resembles that of the sea-anemone *Metridium* described by Grimstone, Horne, Pantin, and Robson (1958). Both acidophil and cyanophil granules pass through the intercellular substance in channels lined only by a plasma membrane. The orientation of the channels is approximately tangential to the pharynx-lumen in cross-section. These channels of secretion granules, together with the intercellular matrix, make up the bulk of the interior region of the pharynx wall. Occasional nerve-fibres and radial muscles are the only other cellular components of this region.

Lining the distal region of the pharynx lumen is an inner insunk epithelium, identical with that which covers the outer surface of the pharynx, and with inner longitudinal and circular muscle-layers situated between its nuclei and the lumen of the pharynx.

Nearer to the base of the pharynx, the nuclei of the epithelium bounding the lumen are not insunk, and the cells display a highly characteristic cytoplasmic ultrastructure. The outer surface is covered with numerous large microvilli attached to a membrane that stains darkly with phosphotungstic acid and is apparently pierced by large numbers of elongate holes, approximately 0·25 $\mu \times$ 0·1 μ, which in longitudinal sections can be seen to be the tips of elongate vesicles or crypts (fig. 8, A, B). These are all oriented with their long axes approximately normal to the cell membrane and form a palisade 0·5 μ to 1·5 μ deep. Beneath this palisade the cytoplasm is still equally highly vacuolated, but the vacuoles vary in size and shape. The appearance of these cells suggests that a massive uptake of fluid might be taking place, but very few mitochondria have been observed. The nucleus lies centrally in the cell.

The gastrodermis of the intestine is surrounded by occasional muscle-fibres, and is composed of two types of cell. The most numerous are large, vacuolate cells containing food in the process of intracellular digestion and with fat deposits at their base. The second type of cell is club-shaped and contains acidophil granules. This confirms the description of the gastrodermis given by Willier, Hyman, and Rifenburgh (1925).

Basement membrane

The epidermal basement membrane in the body of *Polycelis* is thick, varying from 2 μ dorsally and up to 4 μ ventrally. In transverse section it

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FIG. 8 (plate). Preparations fixed with osmium, stained with P.T.A. Sections of inner epithelium of pharynx.

A, transverse sections of two cells showing microvilli, pores in the cytoplasmic membrane, and a cilium. Below the cilium is a sense cell in transverse section.

B, longitudinal section showing microvilli, palisade of vesicles, and highly vacuolated cytoplasm.

shows positive birefringence with respect to its length, and this suggests that it might be formed from elongated protein fibres lying in the plane of the membrane. Pieces of the basement membrane 2 mm square have been isolated by using potassium phosphotungstate. They showed no birefringence except where folded or seen end-on. This suggests that if fibres are present, they are distributed either at random or in a regular orthogonal lattice within the plane of the membrane.

In the electron microscope, the membrane is seen to contain fibrils only 100 Å wide, some of which show a banding of approximately 200 Å interband distance. They are associated with a granular material which sometimes gives the fibrils a pseudostriated appearance or resembles large numbers of fibres cut transversely (fig. 6, A). In longitudinal sections of the membrane (fig. 9, G), some fibrils are grouped parallel to one another, but the orientation of one group bears no relation to the orientation of other groups. Between the groups, the orientation of the fibres appears to be at random in the plane of the membrane. Immediately below the epidermis, the basement membrane consists of densely stained, very fine, branching structures arranged vertically.

The basement membrane is PAS-positive and occasionally stains with alcian blue. Isolated fragments heated to 70 or 80° C do not show the thermal contraction characteristic of collagen, but this may well be due to changes in the fibrils produced by the method of isolation.

The birefringence of the basement membrane in transverse section, and the ability, present in control sections, to stain with aniline blue in the Mallory and Heidenhain's Azan techniques, are lost in sections treated with serologically specific collagenase. It appears that the basement membrane, therefore, contains collagen.

In preparations fixed with osmium, immediately below the basement membrane, are to be found spherical, hollow granules approximately 0·5 μ in diameter. They occur in groups of 6 or more (fig. 6, A, B). Owing to their small size, no routine histochemical tests have been applied to them. They are supposed to consist of lipid, however, since they strongly resemble the 'Ringkörner' investigated by Starke (1895); these are produced by the action of alcohol on osmium-fixed fat globules embedded in tissues. The granules stain very intensely with osmium and appear blue in osmium / ethyl gallate preparations, as does the fat stored in the gastrodermis.

Epidermal epithelial cells

These are essentially columnar cells, 35 μ high on the dorsal surface and 20 μ high on the ventral surface of the animal. Their bases are sculptured to enclose fluid-filled cavities between the basement membrane and the cell. The cavities, which may be seen by both light and electron microscopy frequently extend to the level of the nucleus and may be so extensive that the cell-body is supported on a series of pedestals attached to the basement membrane. Such cavities appear to form a continuous system, ramifying above the basement membrane. They may have a hydrostatic function, as suggested by

Robson (1957) for a similar system in *Metridium*, or they may be associated with the extrusion of granular secretion-products through the cell. The ventral region of the epithelial cells stains strongly with phosphotungstic acid, contains very few mitochondria, and shows negative birefringence with respect to the tangent to the epidermal surface. The outer regions of the epidermal cells adhere closely to each other and are connected by septate desmosomes (Wood, 1959).

All the epidermal epithelial cells contain rhabdites. On the dorsal surface the rhabdites range from 5 μ to 20 μ in length, with a mean length of 15 μ, while those on the ventral surface are smaller and less numerous, averaging 10 μ in length. They are disposed perpendicular to the surface of the cell with their outer ends just below the outer cell surface. There is no cytological evidence that rhabdites are formed in the epidermis, nor is there any connexion between the rhabdites and the basal bodies of the cilia. Such a connexion was postulated by Prenant (1919) but was already questioned by Pedersen (1959*a*).

Rhabdites

Physical properties. The rhabdites are colourless, highly refractile bodies, isotropic in polarized light. They absorb ultra-violet light strongly at a wavelength of about 2,600 Å, but do not fluoresce in the light of a mercury vapour lamp. Their consistency is soft and gelatinous and they appear to be too easily deformed to act as sensory levers, nematocyst analogues, or epithelial supports, as suggested in early theories of their function. They can be stretched to form long threads. Though they adhere to glass, they do not adhere to polythene, and when over a polythene support they can be reversibly extended to twice their normal length; that is to say, they show rubber-like reversible extensibility up to 100% of the resting length.

As shown by Jennings (1957) rhabdites swell when placed in water. Even when fixed in formalin, they swell considerably when stained in azocarmine owing to the acetic acid present. Frozen-dried rhabdites, made insoluble by storing for one day in absolute alcohol after sectioning, lengthen to twice their normal length when placed in acetic buffer solutions below pH 4·5. The number elongating increases as the pH is lowered, until at pH 3 all have elongated. Whether this elongation is accompanied by a decrease in diameter, has not yet been determined since the rhabdites adhere to, and spread over, the glass slide.

Biological properties. Rhabdites are often supposed to form mucus and they are certainly expelled from the epidermis under the influence of noxious stimuli leading to the discharge of mucus. As suggested by Hyman (1951), expulsion might be due to the convulsive contractions induced under these circumstances. In spite of the high ultra-violet light absorption of rhabdites, no u.v.-absorbing substance is detectable in the trail so long as the animal is covered with water.

Chemical properties. The rhabdites are strongly acidophil; Pedersen indeed found them to stain with eosin even at a pH of 11·8. This has been confirmed with eosin, orange G, and methyl orange, with a minimal ionic concentration of buffer; it strongly suggests the presence of arginine, in which the highly basic guanido-group is ionized even at a very high pH. By an unspecified method, Jennings (1957) found that rhabdites have a pH of 8·0 to 8·2. The Sakaguchi test for arginine is positive. The colour developed is quite strong by the modification of Thomas (1950) as described by Liebman (1951); but rhabdites in any case darken in strongly alkaline solutions, and this would give the impression of a darker colour.

The Millon test for tyrosine is very weakly positive, but the xanthoproteic test on Zenker-fixed material stains everything except the rhabdites. This might be due, however, to vacuolization of the rhabdites—a phenomenon often noticed in Zenker-fixed material.

With the Chèvremont and Frederic test for sulphydryl groups and disulphide groups, rhabdites give no reaction. This observation confirms the findings of Brown (1950), who used the same technique. The only objects in the rhabdite-forming cells which react positively are small, circular or oval objects which might be very young rhabdites. Yamamoto (1957), applying the test after Bouin fixation and cyanide reduction, states that the rhabdites blacken; he proposes this as possible evidence for disulphide groups. This effect has been confirmed, but only with Bouin-fixed worms; it is held to result from the interaction of the fixative and the test reagents. Pedersen (1959*a*) found no sulphydryl groups in formalin-fixed rhabdites, but obtained a strong reaction when Zenker-fixed material was used. The use of a mercuric fixative when testing for sulphydryl groups, however, is always contra-indicated.

The periodic acid / Schiff reaction is negative and rhabdites do not stain with toluidine blue.

Prenant (1919) suggested that rhabdites might contain a nucleoprotein, but the Feulgen reaction is negative and they do not stain with methyl green / pyronin. These results confirm those of Pedersen (1959*a*). The absorption of ultra-violet light suggests, however, that purines or pyrimidines are present. Pedersen argues that since the Feulgen reaction is negative, purines cannot be present in the tissue. Purines are in any case removed, however, during hydrolysis with hydrochloric acid in the Feulgen reaction. The ultra-violet light absorption is not diminished by incubation in 3 changes of buffered ribonuclease at pH 6·2 for 2 h at 39° C, with an enzyme concentration of 2 mg/ml (fig. 9, C, D). This suggests that ribonucleic acid is absent, but does not rule out the possibility of the presence of purines. The presence of purines in the rhabdites has been tentatively confirmed by a preliminary study of rhabdites isolated in bulk by homogenizing frozen-dried animals and centrifuging out the rhabdites in a non-aqueous medium (Behrens, 1938), using a density gradient. The rhabdites have a density of 1·29. They were treated with ice-cold 5% trichloroacetic acid, and the resulting supernatant was then chroma-

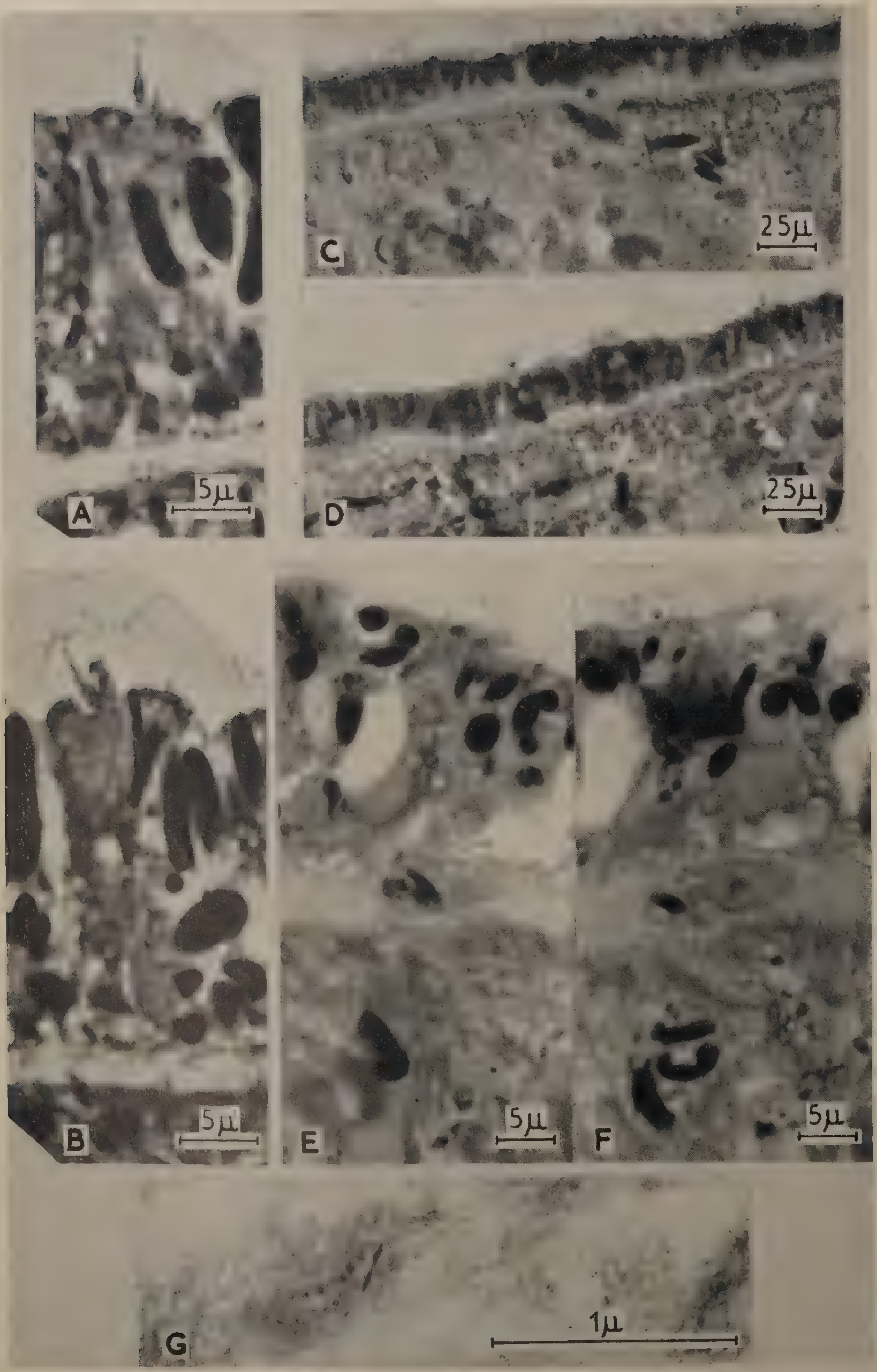

FIG. 9

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tographed, by use of *iso*-propanol and HCl. An ultra-violet absorbing spot was obtained with a mobility of 0·37, corresponding closely to the Rf of adenine.

In spite of their strong acidophilia, rhabdites stain intensely with iron haematoxylin. This anomalous staining was investigated by the method of Wigglesworth (1952), in which metal ions are converted to sulphides. The rhabdites stained strongly with iron, copper, and silver salts, but less strongly with cobalt and lead; the duration of staining had little effect on the intensity. It seems unlikely that rhabdites are sufficiently basic to precipitate the hydroxides of these metals as such, and the fact that the intensity of staining is independent of time, suggests that a complex is being formed, perhaps with the purines as well as with the proteins.

Rhabdites also stain strongly brown with Lugol's iodine; but since this staining is not affected by diastase, it cannot be due to the presence of starch-like polysaccharides.

In rhabdites isolated in bulk by tryptic digestion, Prenant (1919) found calcium to be present, and confirmed this spectroscopically. Calcium salts will form lakes with purpurin, and alkaline solutions of this dyestuff stain rhabdites strongly. This is probably due to acidophil staining rather than lake formation, however, since previous treatment of the section with HCl to remove calcium does not affect the staining. This phenomenon has also been found by Pedersen (1959*a*), with alizarin red and titriplex.

Substitution methods for calcium are equally unreliable owing to the presence of purines, which will complex with the metallic salt used and mask any substitution which may have taken place. Thus von Kóssa's method using silver is positive, as is also the rubeanic acid method using copper.

Micro-incineration shows that there is only a small quantity of minera salts in rhabdites. After incineration, the rhabdite is reduced to a small nodul of ash (2 μ), surrounded by a large clear space which represents the area previously covered by the rhabdite. Although it seems probable that a very small amount of calcium is present in rhabdites, as in other cellular components, this amount is unlikely to be detected histochemically. The large amount of calcium found by Prenant may have been absorbed from the trypsin solution used.

With the Sudan black technique of Baker (1949), rhabdites give a negative result, but they stain strongly with osmium / ethyl gallate. Wigglesworth

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FIG. 9 (plate). A, osmic/gallate preparation. Transverse section of epidermis showing pear-shaped sense organ.

B, osmic/gallate preparation. Transverse section of epidermis, showing clear region of cytoplasm at base of sense organ.

C, frozen-dried preparation. Section digested for 2 h with ribonuclease. Photographed in ultra-violet light at 2,600 Å.

D, control section incubated in buffer at pH 6·2.

E, osmic/gallate preparation. Transverse section of ventral surfaces howing cell with rhabdites within the basement membrane.

F, osmic/gallate preparation. Section adjacent to E.

G, preparation fixed with osmium, stained with P.T.A. Longitudinal section of basement membrane, showing groups of parallel fibres and a mucus-canal in transverse section.

(1957) believes the latter reaction to indicate the presence of phospholipids, but Pedersen (1959*a*) concluded that phospholipids are absent.

Origin. Korotneff (1909) supposed that rhabdites develop in the parenchyma and are later insinuated into the epidermal cells. This idea is also repeated by Pedersen (1959*a*). Substantially the same hypothesis was advanced by Woodworth (1891), but he believed that epidermal rhabdites are situated between the epidermal cells. Prenant (1919) believed that he had evidence that epidermal rhabdites develop in their final situation. No support for the latter theory has been found.

That rhabdites undoubtedly develop in the parenchyma has been shown by following their development in a wound blastema, and as has already been described, there is abundant cytological evidence that rhabdites develop in the rhabdite-forming cells of the parenchyma, for 'young' forms, differing in structure as well as in size from fully-developed rhabdites, have been found. These are associated with a well developed endoplasmic reticulum, with mitochondria, and with piles of flattened vesicles.

It would be unprecedented for an intracellular organelle to leave the parent cell, cross a basement membrane, and become an intracellular organelle in a different cell.

In a survey of the frequency of mitosis in different regions of the animal, many cell-divisions have been observed in the parenchyma but none in the epidermis. This and other observations suggested that the rhabdite-forming cells themselves might move through the basement membrane and thus form the epithelium. Strong support for this idea of a centrifugal migration of rhabdite-forming cells is given by the finding of rhabdite-containing cells within the basement membrane (fig. 9, E, F).

Rhabdites act as useful intracellular markers; and since all the epithelial cells contain rhabdites, it seems likely that in the adult animal, all the cells of the epidermis are derived from the parenchyma and that the epidermis is continually added to by parenchymatous cells traversing the basement membrane to take up position in the epidermis.

Epidermal cilia

These are largely confined to the ventral epithelium of the body and the outer epithelium of the pharynx, but sporadic tufts occur on the inner surface of the pharynx and dorsally on the body. Each cilium exhibits the characteristic set of 11 hollow fibres, 9 of which are peripheral and double, whilst 2 are single and central. The central 2 can be seen to be linked together laterally. Nine finer fibres are sometimes visible, co-radiate with, but internal to the 9 double fibres. Thus the structure of the cilia closely parallels that of the flagella of *Trichonympha* described by Gibbons and Grimstone (1960).

The basal bodies resemble those of *Rana pipiens* (Fawcett and Porter, 1954). The two central fibres are attached to the distal side of an intraciliary septum situated 0·13 μ above the surface of the ciliated cell. Beneath the junction of central fibres and septum is a lens-shaped thickening of the septum.

Below the septum, the diameter of the cylinder formed by the 9 peripheral fibres is reduced, and the fibres join laterally just above the plane of the plasma membrane of the cell to form a ring of 9 hollow tubes. It is not known whether each of these tubes is formed by fusion of the components of the double fibres, or by loss of one of them.

Two striated ciliary rootlets extend into the cytoplasm. One is a prolongation of the basal body, running 2·5 μ into the cytoplasm, and its axis is normal to the cell surface. The other arises laterally from the basal body and lies parallel to the cell surface, but perpendicular to the plane joining the two central fibres of the cilium. Its axis lies at right angles to the plane of beat of the cilium. The periodicity of the rootlet striations varies considerably from fibre to fibre.

Epidermal sense organs

The only sense organ in triclads hitherto described accurately is the eye, though rhabdocoels have been shown to possess other sense organs considered to be rheoreceptors, chemoreceptors, and tangoreceptors. A pear-shaped structure 5 μ long, attached by a thin stalk from its broad (0·7 μ) end to a region of clear cytoplasm within an epidermal cell, has been observed both in living and fixed material (fig. 9, A, B). These structures, of which as many as 6 may occur in a group, have the structure and position to be expected in a rheo- or tango-receptor. They are most numerous in a zone around the lateral edge of the animal, just dorsal to the main ventral ciliation. On the dorsal surface they are very much less numerous, and one has been found protruding into the lumen of the pharynx. In cross-section under the electron microscope they show a central core, 0·13 μ in diameter, formed from 9 fused, hollow tubes; this structure recalls the basal bodies of the cilia. Fibrils extend from the central core into the cortical cytoplasm. The latter is finely granular and is bounded by a plasma membrane.

Discussion

Of the various authors who have considered the structure of the turbellarian parenchyma, many, from the time of Schneider (1873), have regarded it as a syncytial network. Hallez (1879) and Iijima (1884) applied this concept of turbellarian structure specifically to triclads. Prenant (1922) considered that his researches confirmed and supplemented the idea of a syncytial network, interspersed with free cells, and it is largely on this work of Prenant that modern accounts of the triclad parenchyma are based. The reasons for this view, which seems to have arisen from the difficulty of seeing cell boundaries with the light microscope, cannot be ascribed simply to bad fixation, for even when material prepared for the electron microscope is examined by light microscopy, cell membranes are not visible in the parenchyma. In other regions of the body, and in other animals, however, cell boundaries may be seen clearly with the light microscope, in spite of the fact that their dimensions are less than the theoretical resolving power of the instrument. Thus, in *Ascaris*, the gut is very obviously cellular, whilst the hypodermis appears to be syncytial. A high degree of interdigitation between cells might obscure the normal cell limits, but this is not the case in the parenchyma of *Polycelis*; nor do the distances between cell surfaces appear to be any larger in those cells with an optically distinct delimitation, than in those of the parenchyma. A cell boundary visible in the light microscope might be a chemical characteristic of certain cells.

Electron microscopy has made it possible to give an account of the structure of *Polycelis* in terms of different types of component cells, without having recourse to the notion of a syncytial residuum. In general, syncytia may arise by fusion of similar cells or by the incomplete separation of cells formed by simultaneous divisions; in either case, all components of a syncytium are essentially alike. The high degree of local diversification observed in the parenchyma is probably incompatible with a syncytial organization, and the argument for a syncytium is weakened by this very diversity even before we consider the electron microscope evidence for the presence of cell membranes. These are visible with the electron microscope around all the components of the parenchyma, but owing to the smallness of the field scrutinized in electron microscopy, and the necessity for making use of very thin sections, it is difficult to exclude the possibility that a small proportion of cells are binucleate. The demonstration that the parenchyma is cellular, however, simplifies further problems of regeneration, and enables the physiology of the animal to be analysed more precisely. Thus, in the case of the water relationships of *Procerodes*, the syncytial parenchyma has been regarded as a general intermediary between the outer surface and the gut (Beadle, 1934). The actual cells involved could now be established.

The mechanism whereby the necks of the gland-cells of the parenchyma penetrate both the basement membrane and the epidermal cells is not known; nor how a permanent connexion between the gland-cells and the exterior of the animal is maintained despite replacement of the epidermal cells.

The observed structure of the basement membrane differs from that found by Clark and Cowey (1958), who used silver impregnation and light microscopy. They, themselves, found that the living worms did not show the degree of extension which should be possible with a basement membrane structure as they described it. They attributed this fact to the presence of dorso-ventral muscle-fibres. Isolated fragments of basement membrane, however, do not show any great degree of mechanical extensibility when stretched. From their results it is simplest to assume that the membrane has not the regular lattice-like structure of alternating right and left geodesic helices they have proposed for it; and on the basis of polarized light and electron microscopy it appears that the membrane is made up of collagen fibrils with an essentially random distribution in the plane of the membrane.

The apparent renewal of the epidermis by rhabdite-forming cells from the parenchyma invalidates attempts to divide the animal into three distinct and independent layers, as occurs in the germ-layer theory. Similarly, in this animal, it cannot be decided from which of the postulated three layers a given cell type originates. Thus the gland-cells of the parenchyma cannot be considered to be 'epidermal' in origin if the epidermis is parenchymal in origin. It is interesting that the migration is of rhabdite-forming cells, rather than of totipotent, undifferentiated cells which also have the power of locomotion; for it suggests that their passage to the exterior of the animal is associated in some way with the functions of rhabdites. It is also interesting that a more or less spherical rhabdite-forming cell is transformed as a result of the migration, into a cell with a distinct polarity, both with regard to the cell surfaces and also to the oriented contents of the cell.

The histochemical results indicate that rhabdites consist largely of combined arginine and purine; the results of Pedersen (1959*a*), based on a study of their acidophilia, tend to confirm this. Pedersen acetylated and nitrosated rhabdites for considerable periods but found no decrease in their acidophilia. He correctly assumed this to be due largely to the fact that the guanido-base of arginine is only slowly attacked by acetic anhydride and nitrous acid, as mentioned by Olcott and Fraenkel-Conrat (1947). At room temperature, acetic anhydride acetylates primarily the ϵ-amino-group of lysine, and the phenolic hydroxyl group of tyrosine. This is mentioned by Pedersen, but he overlooks the fact that after fixation with formalin, very few of these groups will be available for acetylation, owing to combination with formalin and also the formation of cross-linkages by addition and condensation.

Nitrous acid and hot acetic anhydride, also used by Pedersen, are rather less specific. van Slyke (1911) found that adenine yields the full theoretical equivalent of nitrogen from its amino-group in 1 or 2 h with nitrous acid. Owing to this lack of specificity, some of the amino-group cross-linkages produced by formalin fixation will be split, and basiphil as well as acidophil material, previously fixed, will be rendered soluble. The ensuing loss of basiphil material will be reflected in a continuing acidophilia of the rhabdite in spite of the simultaneous breakdown of the basic groups, for as shown by Alfert and Geschwind (1953), at high pH it is the total balance of charge rather than the number or type of bases present in the protein which determines the staining.

The simplest biological interpretation of the chemical nature of rhabdites is that they are organelles of purine excretion. The fact that while the animal decreases in size during starvation, the ratio of rhabdites to tissue remains unchanged, lends support to the idea of their purine excretory function. As Gontcharoff (1959) has pointed out, the rhabdites of invertebrates strongly resemble, in electron microscope structure, the inclusions in the chloragogenous tissue of annelids.

Though the epidermal sense cells described in *Polycelis* seem well adapted both by shape and position to respond to water-currents, the animal lives in still ponds and ditches where there is no water flow. The sense cells are situated all along the body of the animal; but simply for the detection of mass movement of water, a few at the head end would suffice. The sense-cells are all arranged laterally on the body as though for the unilateral detection of water currents; mass movement of water would be most effectively detected by sense cells present on the dorsal surface. The occurrence of the sense organs in groups, and the facts that they extend laterally all down the body and that each is expanded basally, suggest that they might be analogous to the fish lateral-line system. Support for this idea comes from the fact mentioned by Jennings (1957) that *P. cornuta* responds directionally to water currents set up by other animals nearby. *P. cornuta*, however, has head tentacles and lives in regions of streams with a slight water flow. The only similar reaction which could be provoked in *P. nigra* was a movement away from a nearby source of stimulation provided by a jet of water from a fine pipette directed vertically downwards.

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A Comparison of the Nervous Systems of two Sea-Anemones, *Calliactis parasitica* and *Metridium senile*

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With two plates (figs. 1 and 2)

SUMMARY

The properties of the actinian nervous system are known mainly from physiological experiments on *Calliactis parasitica* (Couch), and from histological work on *Metridium senile* (L.). The structure of the nerve-net in the mesenteries of *Calliactis* is now shown to resemble in general that in *Metridium*. Methylene blue stains a network of bipolar cells over the retractor muscle, together with sense-cells, and unlike *Metridium*, multipolar nerve-cells. The nerve-net over the radial surface of the mesentery is similarly much sparser. The distribution of nerve-cells and sense-cells in the column also resembles that in *Metridium*.

Experiments on *Metridium* show that as in *Calliactis*, the rate of conduction in the mesenteries is greater than in other parts of the anemone. The column, including the sphincter region, conducts more slowly. It is thus shown that the presence of a well-developed nerve-net over the retractors is associated with the development of fast tracts in the through-conduction system, and of rapid, facilitated contractions of the retractor muscles, in both species of anemone.

THE nervous system of sea-anemones has been studied from two points of view. Its physiological properties have been shown by experiment to resemble in many respects those of vertebrate nervous systems (Pantin, 1935), and histological work demonstrates a nerve-net whose arrangement agrees with these properties (Hertwigs, 1879; Pantin, 1952; Batham, Pantin, and Robson, 1960). This paper is about the correlation of the two sources of evidence in the analysis of the rapid responses of *Calliactis parasitica* (Couch) and *Metridium senile* (L.).

THE CLOSURE REFLEX

In the rapid closure reflex of *Calliactis* a specialized part of the conducting system is concerned with the response of sphincter and retractor muscles. Histological evidence that this system is nervous, while supporting the physiological work on *Calliactis*, has been derived largely from a different anemone, *Metridium*. It is thus desirable to assess any differences between the two species in relation to general conclusions about the actinian nervous system. A few observations on the structure of the nerve-net in *Calliactis* and on its rate of conduction in *Metridium* are now reported.

A diffuse, rapidly conducting system innervates the sphincter and retractor muscles in *Calliactis* (Pantin, 1935). It shows absolute and relative refractory periods and chronaxie similar to those of vertebrate nerves. Above threshold,

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frequency of electrical stimulation markedly affects the muscular response, and at shock intervals of less than 3 sec neuromuscular facilitation produces separate, graded contractions. Although 'interneural' facilitation may be demonstrated in certain regions of the anemone, the part of the conducting system which supplies the muscles of the column, the sphincter, and the retractor muscles is continuous physiologically. It has therefore been termed the 'through-conduction system'. In *Calliactis*, physiological experiments show that tracts which run vertically in the mesenteries and join a ring in the sphincter region conduct much more rapidly than the rest of the nervous system (fig. 5, F, p. 324). The through-conduction system may be excited by mechanical stimulation of the column (Passano and Pantin, 1955). A similar through-conduction system exists in *Metridium* (Hall and Pantin, 1937), although it has not yet been shown that fast tracts are present in the mesenteries.

Histological evidence from this anemone (Pantin, 1952; Batham, Pantin, and Robson, 1960) shows that corresponding to the position of rapidly conducting tracts in *Calliactis* a well-developed network of bipolar nerve-cells runs over the retractor surface of the larger mesenteries. Interneural and neuromuscular junctions have been demonstrated, and the processes of abundant sense cells along the junction of mesenteries and column are in contact with the mesenteric nerve-net. Similar but sparser nerve-nets are seen on the radial surface of the mesenteries and in the column.

These two lines of investigation suggest that the development of a rich network of bipolar nerve-cells is associated with the development of fast conduction and of rapid, facilitated contractions of muscles concerned in the closure reflex. In order to confirm this correlation, histological evidence of nerve-cells in the mesenteries of *Calliactis*, and physiological evidence for rapid through-conduction in the mesenteries of *Metridium* are required.

THE NERVE-NET IN *CALLIACTIS PARASITICA*

The nerve-net of *Calliactis* has been demonstrated by vital staining with methylene blue. Large anemones, allowed to expand in the dark for 2 or 3 h, were anaesthetized by transferring to approximately 0·15% propylene phenoxetol (Owen, 1955; Owen and Steedman, 1958). This was prepared by shaking the liquid with seawater in a test-tube, and stirring the emulsion into the main volume of sea-water. The medium was pipetted into the coelenteron at intervals, and large specimens were fully anaesthetized after 3 or 4 h. Staining was carried out at the same time by injecting reduced methylene blue into the

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FIG. 1 (plate). Preparations of *Calliactis* mesenteries stained with methylene blue and fixed in Susa / phosphomolybdic acid.

A, part of the nerve-net over the retractor muscle, whose fibres run from left to right in the background.

B, a bipolar nerve-cell from the retractor nerve-net, with which the fine neurite of another cell makes several lateral contacts.

C, part of the retractor nerve-net, showing a neurite crossing numerous others. Underlying retractor muscle fibres run from left to right.

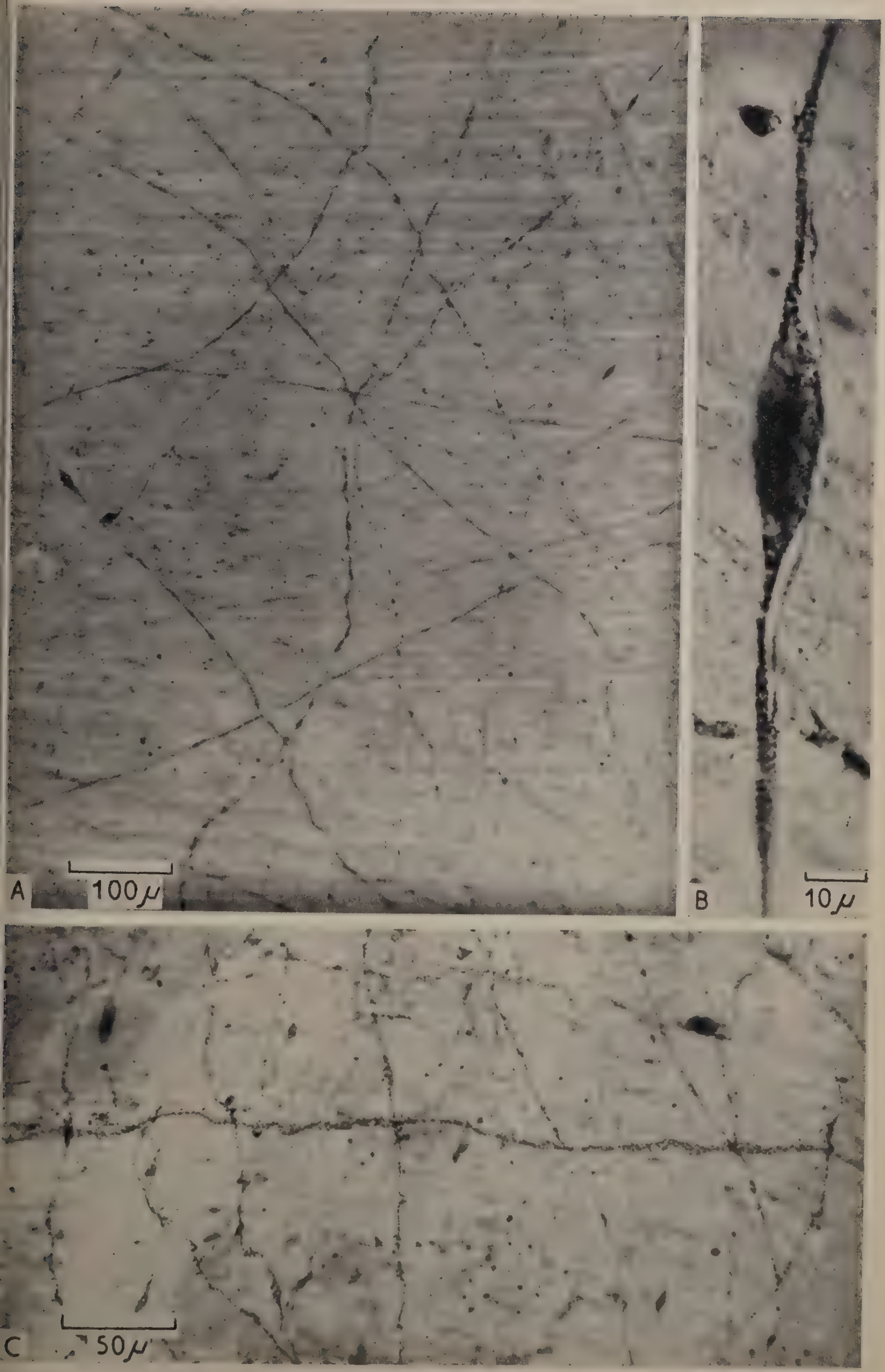

FIG. 1

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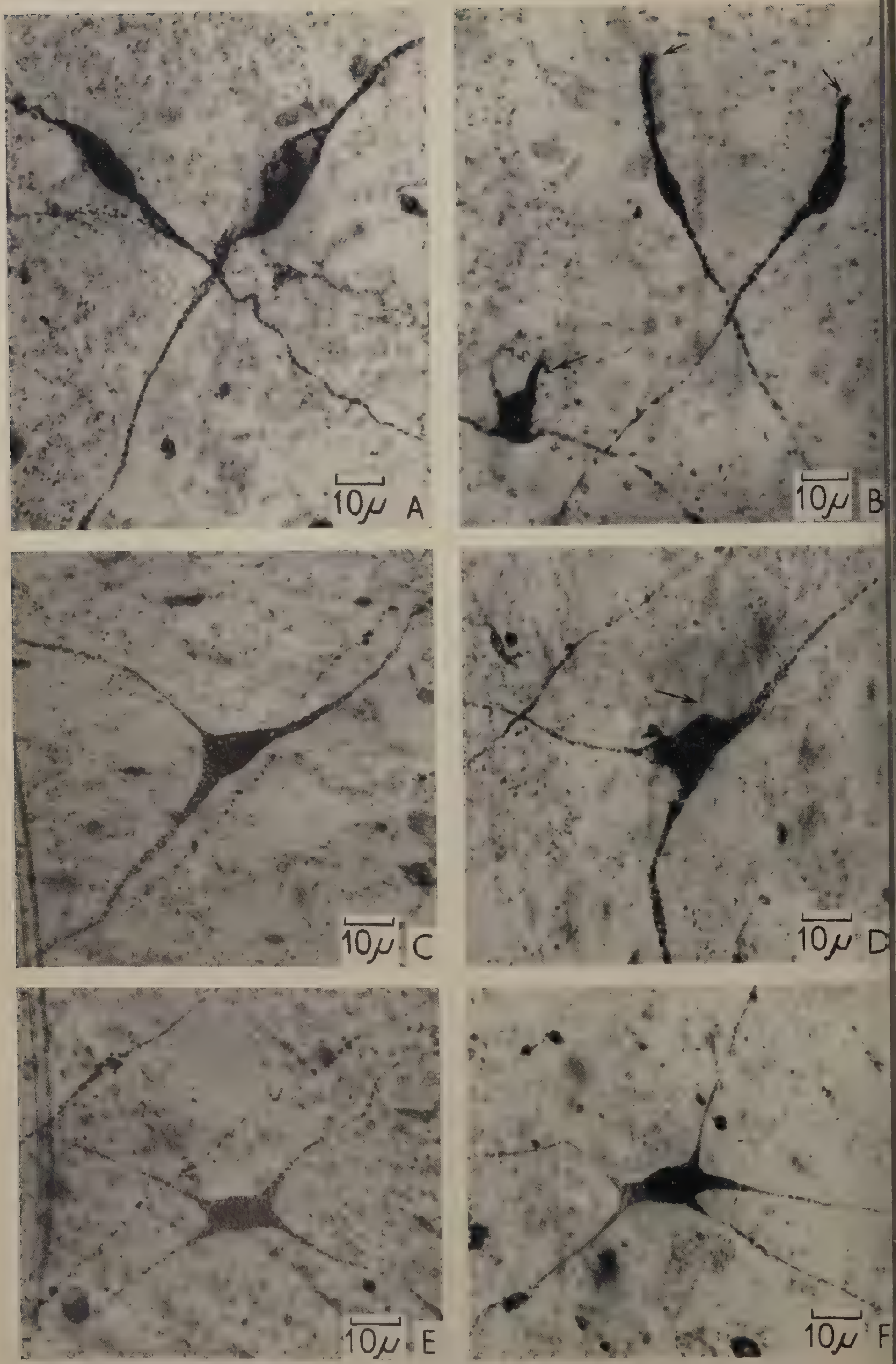

FIG. 2

E. A. ROBSON

coelenteron an hour or two after the anemone was transferred to the anaesthetic. The most successful results were obtained after the dye had been diluted about 5 times with sea-water beforehand. Stained mesenteries and other parts of the anemone were then dissected out, pinned on to wax strips, fixed, and mounted in depex. Mesenteries were fixed in Susa/phosphomolybdate (Batham, Pantin, and Robson, 1960), which precipitates the dye completely although rather coarsely, and thicker preparations with ammonium molybdate.

The retractor surface of the mesenteries has a well-developed nerve-net (fig. 1, A) which is clearly similar to that of *Metridium*. It is composed mainly of bipolar nerve-cells (fig. 1, B), which cross one another at frequent intervals (fig. 1, C), and sometimes tend to run in the direction of the muscle-fibres. The nerve-net does not follow the pleated arrangement of the retractor muscle (Batham and Pantin, 1951; Batham, Pantin, and Robson, 1960) but is situated in one plane below the epithelium. Individual neurites may, however, sink down towards the muscle as in fig. 3. The overall length of bipolar nerve-cells in moderately extended mesenteries is difficult to determine in these preparations, but usually appears to be about 4 or 5 mm. Although they seem to be shorter than the bipolars in silver preparations of *Metridium*, which average 4 to 8 mm (Batham, Pantin, and Robson, 1960), a few cells 7 or 8 mm long suggest that the size ranges are probably similar. The shorter neurites may be incompletely stained in these preparations.

Relatively few nerve-cells are present on the radial surface of the mesentery, but as in *Metridium* their size range corresponds to that of the retractor nerve-cells. The numerical ratio of cells on the two surfaces cannot be measured in these preparations, but as in *Metridium* it is probably less than 10 to one.

Junctions between nerve-cells are numerous (fig. 2, A), although the region of synaptic contact appears in most cases to be more restricted than in *Metridium*. This might be due to the use of a different anaesthetic, since magnesium chloride, which was not used for *Calliactis*, might well affect the cytoplasm and cell membranes in such a way as to increase the area of adhesion. The main observation is, however, that in neither anemone is the nerve-net syncytial. *Calliactis* preparations show lateral contacts between adjacent neurites as in *Metridium* (fig. 1, B) (Batham, Pantin, and Robson, 1960). No new structural features have been observed.

The endings of neurites are difficult to trace in methylene blue preparations,

---

FIG. 2 (plate). Preparations of *Calliactis* mesenteries stained with methylene blue and fixed in Susa / phosphomolybdic acid. All from the retractor surface, and at the same magnification as fig. 1, B.

A, cross-over junction between two bipolar nerve-cells.

B, three sense-cells, 2 with one neurite and one (lower left) with 3, to show junctions between these processes. Sensory cilia are not in focus but their position is indicated by arrows.

C, tripolar nerve-cell.

D, tripolar sense-cell, with the base of the faintly stained cilium indicated by an arrow.

E, multipolar nerve-cell, one of whose 4 neurites makes contact with at least one of the 2 others crossing the photograph.

F, multipolar nerve-cell with 5 neurites.

and have not yet been seen in *Calliactis*. In *Metridium*, their termination over muscle-fibres is best studied in silver preparations of fixed mesenteries.

As described by the Hertwigs (1879), *Calliactis* differs from *Metridium* in possessing multipolar nerve-cells with 3, 4, or 5 neurites, which make contact with the bipolar network. Examples from the retractor nerve-net are shown in figs. 2, C, E, F. Only occasional tripolar nerve-cells are found in the mesenteries of *Metridium* (Pantin, 1952). Sense-cells, differentiated by a short cilium (fig. 2, D), are present in the same preparations. Their processes make contacts with other sensory neurites and with the nerve-net (fig. 2, B, D). The sense-cells may possess 1, 2, or 3 neurites. These may exceed 500 μ in length, but they taper away to fine endings which are no easier to trace in *Calliactis* than in *Metridium*. Sense-cells occur on both surfaces of the mesenteries and may equal a nerve-cell body in size.

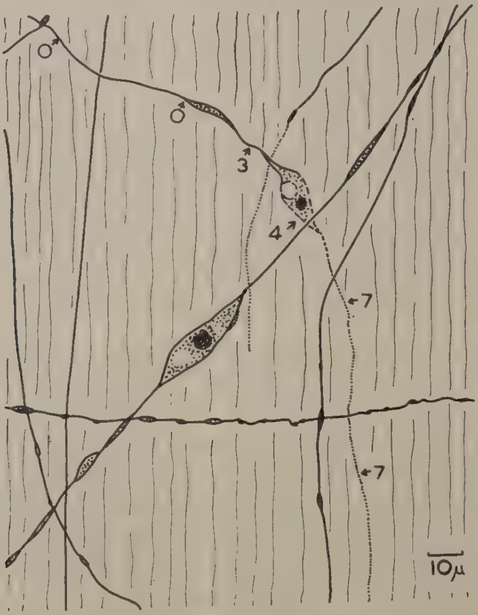

FIG. 3. Drawing to show how the neurite of a bipolar nerve-cell may drop down from the general level of the nerve-net to the folds of the retractor muscle. Numbers indicate distance below general level of focus in μ. (Camera lucida from fig. 1, A.)

A few observations on the column and pedal disk show that these regions are similar to their counterparts in *Metridium* (Batham, Pantin, and Robson, 1960). Numerous sense-cells line the angles where mesenteries join the column and pedal-disk, and their neurites connect up with the retractor nerve-net. The presence of this system in *Calliactis*, inferred from mechanical stimulation experiments, is thus confirmed.

The distribution of the relatively few bipolar nerve-cells in the column is summarized in fig. 4. They often pass beneath the attachment of a mesentery within bundles of circular muscle-fibres, or else turn through 90° on reaching its insertion and either run along the junction or on to the surface of the mesentery. A few run wholly along the base of the mesentery. Sensory neurites may follow these pathways as well.

Multipolar nerve-cells, apparently absent from this region in *Metridium*, have also been seen in the column.

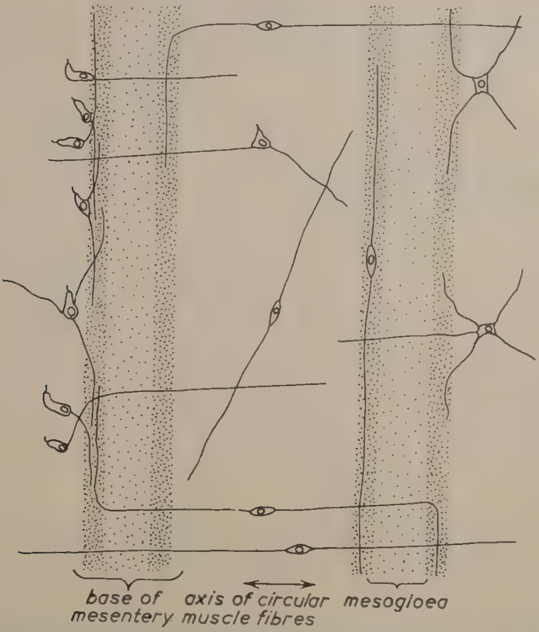

FIG. 4. Diagram to show distribution of nerve-cells and sense-cells in methylene blue preparations of the column of *Calliactis*. The bases of two mesenteries, shown by stippling, are traversed by neurites which accompany bundles of circular muscle-fibres. Only a few of the sense-cells in the angle of the mesentery are shown.

THE THROUGH-CONDUCTION SYSTEM

In *Calliactis* two muscles, the sphincter and mesenteric retractors, are able to give quick, facilitated contractions. The associated fast tracts of the through-conduction system have been shown experimentally to run in the mesenteries and in the sphincter region. The corresponding histological picture in this anemone now shows an extensive nerve-net over the retractor muscle and a similar but much sparser one on the radial surface of the mesentery, resembling the situation in *Metridium*. The innervation of the sphincter is unfortunately not yet known in either anemone.

In *Metridium*, in contrast to *Calliactis*, the retractor muscles withdraw the oral disk before the sphincter contracts (Pantin, 1935 *b*). Both give facilitated contractions, but the retractor muscle is in this case the more rapid of the two (Batham and Pantin, 1954). This agrees with the degree to which the nerve-net is developed over the retractors compared to the radial muscles and column (Batham, Pantin, and Robson, 1960). A few measurements now show that the rate of vertical conduction in the mesenteries exceeds that of any other region, confirming what has been found in *Calliactis*.

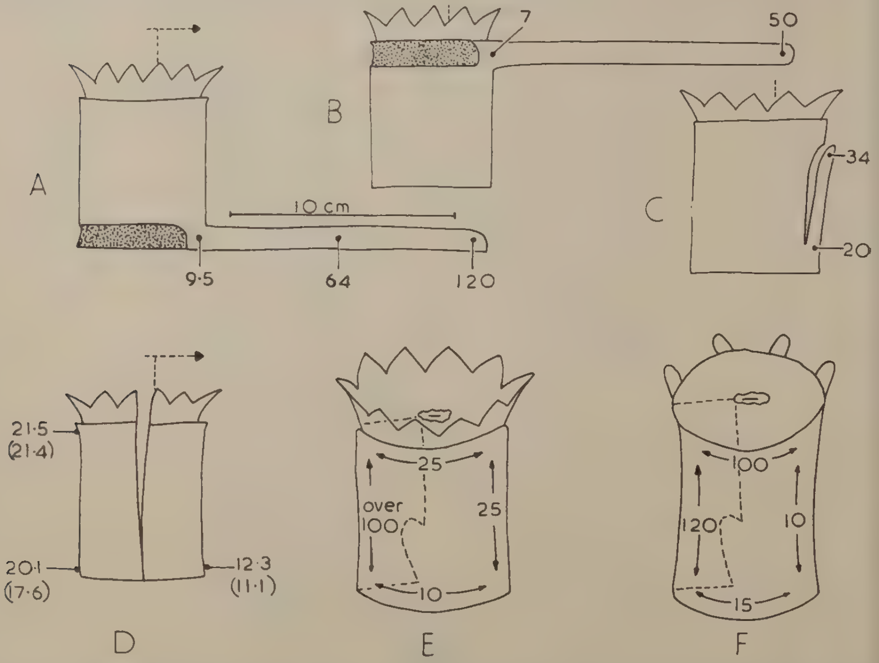

Fig. 5. A–D, diagrams of a few experiments on *Metridium*, showing latent periods in hundredths of a sec. The numbers represent time between the second of two electric shocks and contraction of retractor muscles recorded from a thread in the siphonoglyph. In D measurements from a different specimen are given in brackets. E, diagram showing approximate speeds of conduction in *Metridium* based on present results. F, diagram showing the comparable values in *Calliactis*, after Pantin (1935*b*).

Speeds of conduction were estimated from the latency of response to stimuli from silver / silver chloride electrodes in various positions (Parker, 1918; Pantin, 1935 *b*). Large *Metridium* (4 to 6 cm in diameter) were allowed to settle on glass plates, and any necessary incisions made after anaesthetizing with magnesium chloride (Batham, Pantin, and Robson, 1960). After the preparation had recovered in running sea-water, contractions of the retractor muscles were recorded by a thread through the top of the siphonoglyph. Condenser shocks were given by a slightly modified version of the stimulator used by Hall and Pantin (1937).

Two shocks, 1 sec apart, were given with the cathode in different positions. The latent period between the second shock and contraction of the retractor muscle was then estimated from kymograph records. Although a light spring lever was employed, at the drum speed used (22 cm/sec) the beginning of a contraction was not always clearly defined on the record. Errors due to this factor and to other inadequacies of recording technique are nevertheless small. Variation between different anemones, or in the same individual at different times, may also affect measurements of latent periods, but this similarly does not affect the order of general conclusions.

Fig. 5 summarizes the results. Latent period measurements were obtained in the column and sphincter from attached strip preparations. From these, speeds of conduction were calculated. Owing to the way in which the tissues of *Metridium* contract, the position of the cathode could only be measured to the nearest cm or so, and conduction velocities given in fig. 5, E are approximate. The results obtained nevertheless enable speeds of conduction in different parts of the anemone to be compared.

Speeds of vertical conduction in the mesenteries were obtained from animals divided vertically to the base, by stimulating at the top and bottom of the column on the side away from the recording thread (fig. 5, D). It is certainly higher than anywhere else in the anemone, and may perhaps exceed that of the fast tracts in *Calliactis*. The fact that the sphincter conducts no more rapidly than a vertical strip of column is unexpected by comparison with *Calliactis*, but agrees with the observed slower speed of the sphincter response in *Metridium* (Pantin, 1935 *b*). It may thus be concluded that in both the anemones considered here, rapid vertical conduction in the mesenteries is associated with a well-developed retractor nerve-set, as compared to slower conduction on a sparser nerve-net in, for example, the column.

It may finally be noted that few properties of the actinian nerve-net have so far been correlated with its structure (but see Batham, 1956). It is not known, for example, how synaptic junctions between nerve-cells in a through-conduction system differ from those showing 'interneural' facilitation, although preparations such as that shown in fig. 1, C suggest that a retractor neurite may cross 50 others in the course of 1 mm; or whether nerve-endings differ in the sphincter and retractor from those in muscles which cannot give quick, facilitated contractions, or whether sensory neurites may innervate muscle-fibres directly as suggested by Parker (1919). The function of multipolar nerve-cells, if different from that of bipolars, is not known, and nothing is known of the histology of the sphincter region. It is therefore encouraging that in at least two different species of anemone, existing evidence supports the same conclusions.

I should like to thank the Director and staff of the Marine Biological Association Laboratory, Plymouth, for their hospitality; Professor C. F. A. Pantin, F.R.S., for helpful criticism and discussion; and Mr. K. C. Williamson for assistance with the photographs.

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The Coelomic and Peritoneal Cell Systems of some Sabellid Polychaetes

By R. PHILLIPS DALES

(*From Bedford College, University of London*)

With three plates (figs. 4, 5, and 11)

SUMMARY

The growth and activity of the coelomic cells of *Sabella spallanzanii* are described. Qualitative and quantitative determinations were made throughout the year of the glycogen, oil, and pigments which they contain. The coelomic cells accumulate carotenoids, including astaxanthin. The possibility of the interconversion of glycogen and oil by the cells is discussed, and it is concluded that the coelomic cells constitute a depot for the maturation of the gametes. This system is paralleled in some other sabellids by the development of a villous peritoneum. Such tissues contrast with the chloragogen tissue in that they have no direct contact with the blood vascular system.

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THE PERITONEUM AND COELOMIC CELLS IN SOME OTHER SABELLIDS . . . 339
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INTRODUCTION

If an incision is made into the body-cavity of a large *Sabella* (= *Spirographis*) *spallanzanii* (Viviani), the contents of the coelom will be seen to be bright orange or brown and to have a rich creamy consistency. This appearance and quality is due to a large quantity of free cells. Similar, though smaller and rather less numerous cells may be seen also in *S. pavonina* Savigny and in *Myxicola infundibulum* (Renier). In *Eudistylia polymorpha* (Johnson), *E. vancouveri* (Kinberg), *Schizobranchia insignis* Bush and *Pseudopotamilla occelata* Moore, there are very few cells lying freely in the coelom. Dissection of these species reveals that the peritoneum, unlike that of *Sabella*, is elaborated into villi. These are oily and have a bright orange or brown colour, very reminiscent of the coelomic cells of *S. spallanzanii*. In *S. pavonina* the quantity of cells in the coelom increases with the onset of sexual reproduction, while *S. spallanzanii* contains large quantities of cells at all times. This paper

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represents the result of an investigation of the nature and function of these free cells and of the peritoneum.

Specimens of *S. spallanzanii* were collected in the Bay of Naples and off Monaco and Santander by divers. The *E. polymorpha* studied were obtained from rock crevices under 25 to 50 feet of water, 3 miles north of Point Dume at the western end of Zuma Beach near Los Angeles. *Schizobranchia* was collected from floating wharves near Friday Harbour in Puget Sound, Washington, and *E. vancouveri* was also obtained there and by dredging in the Sound. All the *S. pavonina* came from the Kingsbridge estuary in Devon, where some of the *Myxicola* were also collected; other specimens and also *Pseudopotamilla* were dredged in Puget Sound.

THE COELOMIC CELLS OF *SABELLA SPALLANZANII*

Preliminary remarks

The cells which occur in the coelom of *S. spallanzanii* are rounded and vary from 5 to 70 μ in diameter; in a large worm most are from 45 to 55 μ across. Such cells contain numbers of transparent, oily globules, some as much as 10 μ in diameter, and a central nucleus. Orange and yellowish-brown granules occur between the droplets in the granular cytoplasm. Cells occur only in the coelom; none are to be seen in the blood.

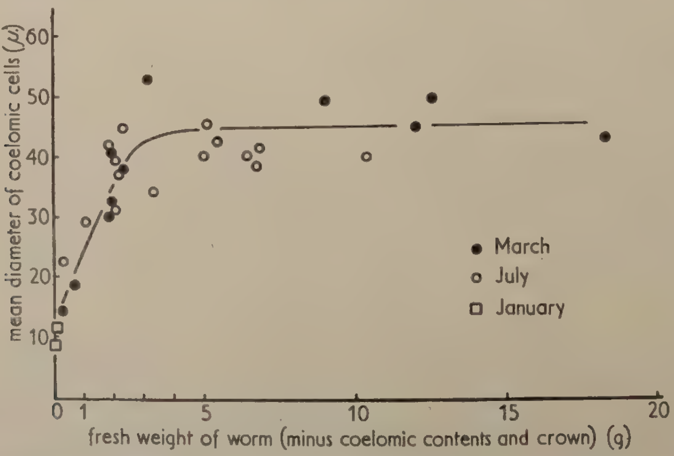

FIG. 1. *S. spallanzanii*. Relation between the mean size of the coelomic cells and the size of the worm. Each point represents the mean of 100+ measurements.

In very young worms the mean size of these cells is a function of the body-size, but those with a fresh weight of 5·0 g or more (excluding coelomic contents and crown) have cells with a mean diameter of 45 μ (fig. 1). Measurement of random samples of 100 or more cells taken from worms of different sizes shows that in worms 5 mm or so in length (fresh weight, 0·003 g) all the cells may be less than 20 μ in diameter, but that large worms (fresh weight, 18·0 g) have hardly any as small as this (fig. 2).

The coelomic cells are strongly phagocytic, the smallest being the most active, though even the largest may occasionally be seen to put out long pseudopodia and engulf free oil-globules. The pseudopodia are extended as long slender processes expanding at the tip, the capture of small oil-globules giving the impression of a throw-net or seine in slow motion. Smaller cells when active often become somewhat spindle-shaped or bipolar, extending their slender pseudopodia in both directions at once. Larger cells almost replete with oil and other inclusions do not lose their globular form when extending a pseudopodium, which is sharply differentiated from the outline of the rest of the cell (figs. 3, A–D; 4, A).

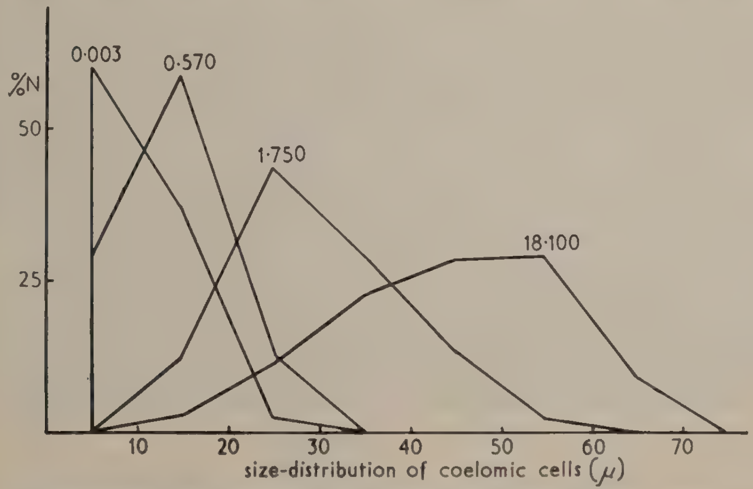

FIG. 2. *S. spallanzanii.* Size-frequency distribution of the coelomic cells in 4 worms of widely different weight. The original measurements were made in 10-μ groups, the number in each group being expressed as %N, where N = 100+ in each case.

All the cells in the coelomic fluid are of this type, and there is no doubt that the smallest and most active cells are continuously taking up particles suspended in the coelomic fluid until they reach maximal size; all intermediate conditions may be seen, and measurement of random samples of cells from any worm shows a normal size-frequency distribution. The largest cells, perhaps for purely physical reasons, seem rather inactive. Disintegration of these cells liberates the contents into the coelomic fluid, the freed granules being then taken up by smaller cells. This appears to happen normally.

The greater phagocytic activity of the smaller cells often causes clumping. Balls of from a few to many hundreds of cells occur in the coelom of some worms, and these masses may be conspicuous by their greater concentration of colour (figs. 3, A; 4, B).

Histochemistry

Staining with haematoxylin and eosin, Azan, and other routine stains, after fixation with various alcoholic fixatives, demonstrated that even the largest cells retain the nucleus, and that the cytoplasm contains numbers of protein granules.

Staining with Best's carmine against diastase controls also showed that large quantities of glycogen in the form of granules about 1 μ across were, present.

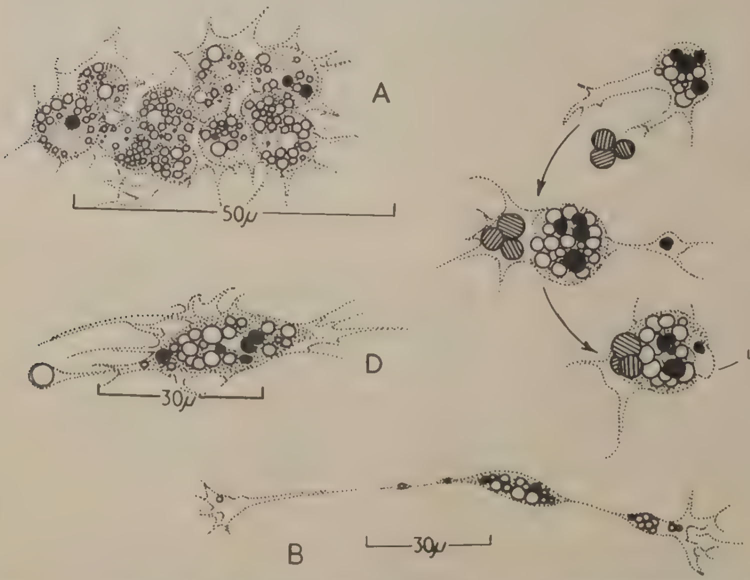

FIG. 3. *S. spallanzanii*. Sketches of living coelomic cells. A, a clump of young cells. B, a young cell putting out long pseudopodia in both directions and capturing oil-globules in the terminal protoplasmic expansions. C, D, similar cells in other characteristic attitudes when taking up oil.

Smears were fixed in Baker's formaldehyde / calcium. They were coloured with acetylated Sudan black B (Casselman, 1954), dissolved in propylene glycol (Chiffelle and Putt, 1951). Controls were made by using hot pyridine or a 2:1 mixture of chloroform and methanol as lipid solvents (compare Fredricsson and others, 1958). The results established the lipid nature of the globules.

The coloration by Cain's Nile blue method (1947) of smears fixed in the same way suggested that the globules contained phospholipid; all the droplets stained sharp blue both with 1% and with 0·02% Nile blue, and no granules of purely neutral fat were detected. Menschik's (1953) Nile blue technique also indicated substantial quantities of phospholipid, as there was little difference between unextracted smears and those extracted with cold acetone. Baker's acid haematein method against hot pyridine controls confirmed the presence of phospholipid in all the lipid globules.

FIG. 4 (plate). *S. spallanzanii*. A, a large coelomic cell, almost replete with oil, extending a pseudopodium (living, phase contrast)

B, a ball of young, actively phagocytic coelomic cells (living; phase contrast).

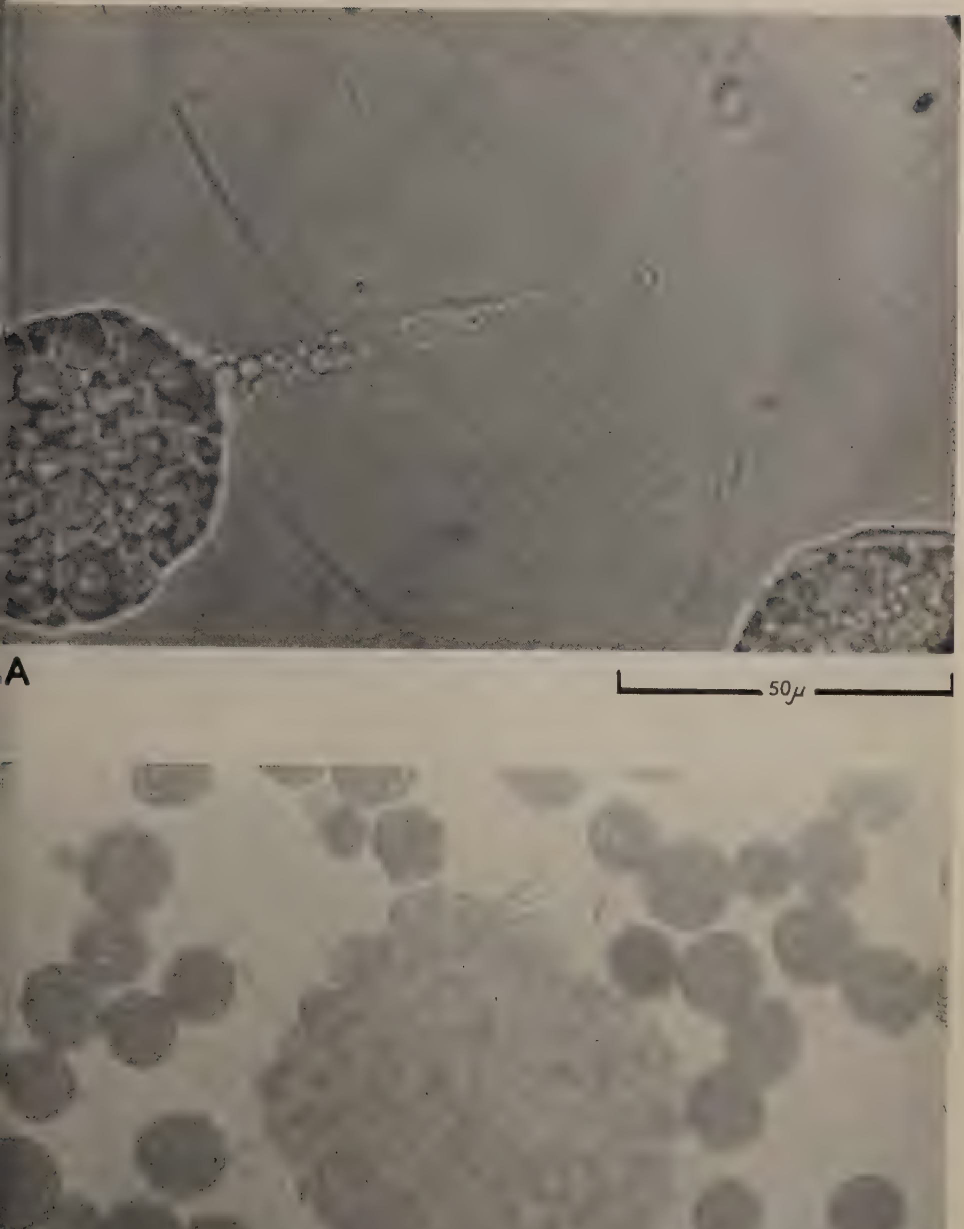

Fig. 4

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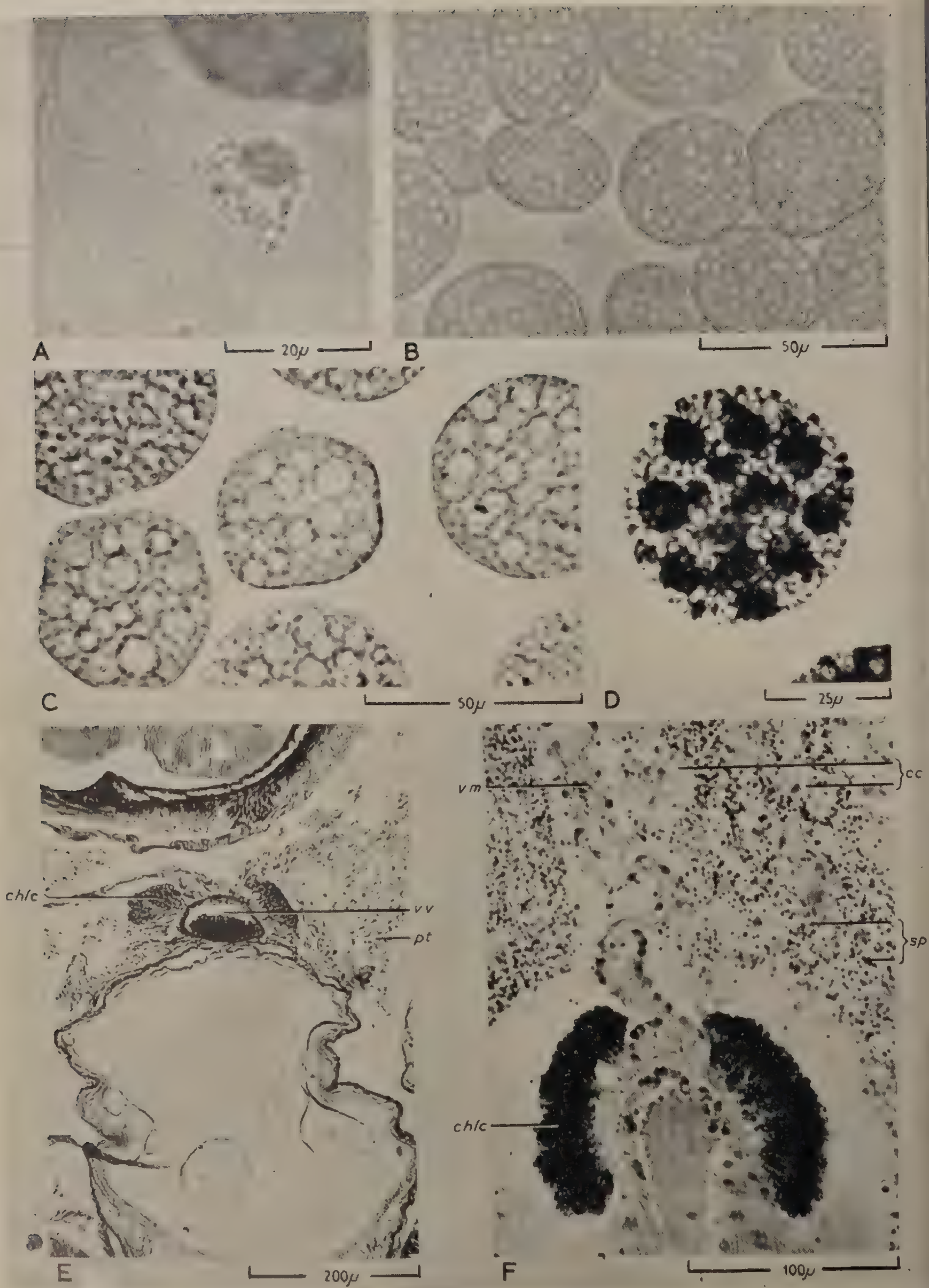

FIG. 5

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The protein granules lying between the globules of lipid and granules of glycogen were unaffected by these colouring agents for lipids (fig. 5, A–D).

Pigments

The body colour of *S. spallanzanii* varies from a bright orange to brown, and the colour of the coelomic cells generally corresponds to that of the body. The oil in the coelomic cells has a faint yellow colour, and the bright orange pigment granules lie separately in the cytoplasm. The colour of the coelomic contents may also be modified by the gametes: the oocytes when mature are turquoise blue, the spermatozoa white, so that the coelomic contents of females about to spawn is green, of males cream to mushroom. The pigments in the oocytes are considered further below. Most of the pigment in the cells is bright orange and was found to be carotenoid; most of the brown pigment in the cells is chromolipid, judging from its solubility and reaction to colouring agents for lipids.

Cells were centrifuged, washed repeatedly with sea-water, and suspended in distilled water, when a light greenish pigment dissolved out. The rinsed cells were extracted with acetone, and the dissolved carotenoids taken into light petroleum (b.p. 40–60° C) and saponified by heating with 12% methanolic KOH under reflux. Extensive saponification was necessary in order to obtain good chromatograms owing to the quantity of oil present in the crude extract. The carotenoids were taken into diethyl ether by the addition of water to the saponification mixture, evaporated to dryness, and redissolved in light petroleum. Separation with 90% methanol showed that most of the carotenoid was epiphasic. The epiphase was chromatographed on alumina and MgO, the column being developed with mixtures of acetone and light petroleum; the hypophase was returned to light petroleum, dried with anhydrous sodium sulphate, and chromatographed on icing sugar (Tate and Lyle, containing 1½% calcium phosphate), the column being developed with mixtures of *n*-propanol and light petroleum.

The epiphase when chromatographed on alumina exhibited the bands shown in fig. 6. The hypophase showed a narrow pink band right at the top of the column similar to band 5 of the epiphase, and at least one yellow band

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FIG. 5 (plate). A–D, *S. spallanzanii*. A, a young, very actively phagocytic coelomic cell with some granular inclusions, mainly carotenoid (living; phase contrast).

B–D, replete coelomic cells.

B, living, phase contrast.

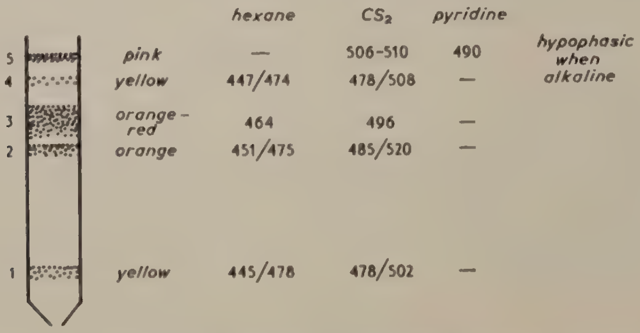
C, Best's carmine—granules of glycogen show as sharp black patches around the transparent oil globules.

D, acetylated Sudan black B—oil-globules appear black; the rounded white granules are the albuminoid granules referred to by Kollman.

E, *Myxicola infundibulum*. T.S. through the nerve-cord and ventral vessel (*vv*) showing the pigmented chloragogen cells (*chlc*) and proliferating bands of peritoneal tissue (*pt*). 10µ paraffin section, stained Azan.

F, *S. pavonina*. T.S. through the ventral vessel (*vv*) showing the pigmented chloragogen cells (*chlc*), ventral mesentary (*vm*), and numerous free coelomic cells (*cc*) amongst the maturing sperm (*sp*).

beneath it, with lutein-like absorption spectra. The pink band at the top of the column resembled astaxanthin. Its occurrence is interesting as it has not before been reported from annelids. After elution the pigment showed the hypophasic (alkaline) / epiphasic (acid) behaviour when partitioned with 90% methanol characteristic of the compound, and was difficult to elute from the column. The appearance of astaxanthin in the epiphase even after considerable saponification of the original mixture suggests its presence in the cells as an ester. Bands 2 and 3 of the epiphase were difficult to separate on alumina and although a somewhat better separation was achieved with magnesium oxide, it was difficult to determine the proportions of each, the mixture giving maxima between 485 mμ and 496 mμ in carbon disulphide on different occasions. Careful and slow elution usually enabled the two components to be separated, the lower band having maxima characteristic of β-carotene, the upper redder band retaining a single absorption maximum about 496 mμ in carbon disulphide, 460 mμ in light petroleum. A mixed chromatogram of this upper band with β-carotene from fresh carrot extract, separated into two bands, the β-carotene remaining below. In the presence of astaxanthin as a pink band at the top of the column it seems probable that the upper red band represents a keto-carotenoid leading to astaxanthin. The general chromatographic picture is, indeed, very reminiscent of Nicola's (1954) analysis of the carotenoids in the integument of the echinoderm, *Ophidiaster*. While in the cells from *S. spallanzanii* there is less astaxanthin than the suspected keto-form, it may be that these worms are able only partially to affect the transition, or that this occurs slowly, so that the keto-form accumulates.



		hexane	CS_2	pyridine	
5	pink	—	506–510	490	hypophasic when alkaline
4	yellow	447/474	478/508	—	
3	orange-red	464	496	—	
2	orange	451/475	485/520	—	
1	yellow	445/478	478/502	—	

FIG. 6. *S. spallanzanii.* Separation of epiphasic carotenoids extracted from the coelomic cells (alumina).

Analysis of the carotenoids in the body-wall and in the crown showed the same series of carotenoids to be present in each, and in much the same proportion. These analyses were repeated many times, with worms from different localities, always with the same results.

The cells extracted with acetone retain a slightly greenish yellow colour. If they are then suspended in distilled water, a greenish solution is slowly obtained. A deep green solution is instantly produced with slightly acidified methanol. The green pigment is insoluble in light petroleum, ether, or chloroform; it gives a negative zinc bilipurpurin reaction, and is unaffected by boiling or by oxidizing agents. In 1% HCl:MeOH it shows an absorption spectrum with a peak at 330 mμ, a shoulder at 380 mμ, and a broad hump at 685 mμ (fig. 7). Addition of vigorous reducing agents such as potassium borohydride to the acid solution reduces the solution to a yellow colour, the peaks at 330 mμ and 380 mμ remaining, that at 685 mμ disappearing. After standing for some time the pigment returns to its former stable green colour. Addition of strong ammonia to the acidified methanolic solution turns the solution to a permanent yellow colour with the development of a strong yellow-green fluorescence in u.v. light. The pigment may be a flavin, possibly linked to a protein.

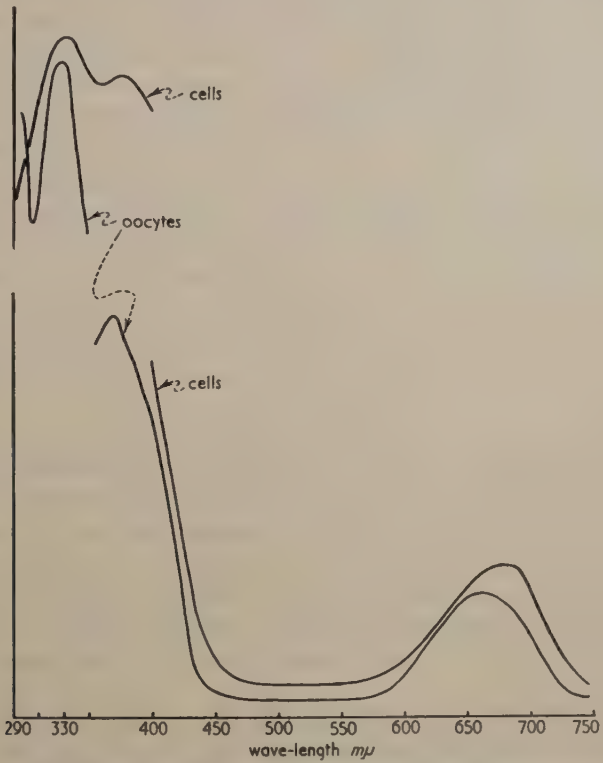


FIG. 7. *S. spallanzanii.* Absorption spectra of fresh unsaponified extracts of the coelomic cells and of the oocytes in 1% HCl in methanol.

By extracting all the carotenoid from the coelomic cells, which were then dried and weighed, an attempt was made to find out whether there was any clear correlation between the total carotenoid and the total dry weight of the cells, but the results were too variable to show any close relationship. A quantitative extract from the rest of the body of the same worms, however, indicated that more carotenoid was deposited in the coelomic cells than in the remainder of the body, and that the concentration of the pigment in the cells was also higher (table 1, see appendix).

The pigments in the oocytes

When mature the oocytes are greenish blue. If suspended in sea-water and boiled they are turned bright yellow. Addition of alcohol or acetone also immediately effects a similar change with the production of a yellow colour which was found to be due mainly to carotenoid. These facts suggest that carotenoid present in the oocytes is in the form of a blue-green carotenoprotein. A deep green pigment may be extracted from fresh oocytes with distilled water (pH 5·5 to 6·0). This was purified by adsorption on to alumina gel, when it appears blue. The adsorbed pigment was centrifuged, washed, and then suspended in 5 to 8 volumes of 0·2 m Na_2HPO_4, when all the pigment came into solution. The colour (pH 6·1) was not, however, destroyed by boiling, nor was it affected by acetic acid, acetone, or light petroleum. The pigment appeared to be rather stable to light and air. Addition of ammonia immediately turned the colour yellow, with the development of a green fluorescence in u.v. light. The fresh oocytes also yielded a deep green solution with 1% HCl:MeOH, and this gave the same reactions as the similar extract obtained from the cells with maxima at 330 mμ, 370 mμ, and 660 mμ in the same solvent.

After extraction with water the eggs are purple. Treatment with acetone produced a yellow colour which was found to be due to carotenoid, the purple colour presumably being due to a carotenoprotein. The eggs remain blue; this pigment has not been identified. Treatment of the fresh oocytes with alcohol or by heat immediately turns them yellow, probably as a result of liberation of the carotenoid. An acetone extract when taken into light petroleum and chromatographed on alumina as before, showed that the same series of both hypophasic and epiphasic pigments were present as have been found in the coelomic cells.

Analysis of the oil in the coelomic cells

In order to characterize the oil extracted from the coelomic cells more precisely, several of the more common procedures used in the analysis of fats were carried out. The oil used for these determinations was that extracted with 2:1 $CHCl_3$:MeOH and purified for the gravimetric estimation described below. All the oil was redissolved in diethyl ether and kept in a tightly stoppered amber bottle under nitrogen in the ice-chest until sufficient had been collected to perform one of the estimations to be described. The ether was then distilled off in a vacuum, the oil dried for 2 or 3 h at 90°, and then left in a nitrogen-filled desiccator.

The oil has a golden colour. It rapidly oxidizes and hardens in air and has the general properties of a drying oil. At room temperatures (18° to 20°) a small fraction crystallizes out, but most remains liquid.

All of the following determinations were done on oil extracted from worms collected in March and April at Naples; the determination of the proportion of C_{20-24} acids was done on extracts made in July. No analyses have been made on oil extracted from other parts of the body.

Proportion of phospholipid. With Bloor's methods for blood-lipids (Bloor, 1928, 1929) as described in an earlier paper (Dales, 1957), it was found that from a single determination of a sample containing 28·15 mg total fatty acid, 5·09 mg or approximately 18% was in the form of phospholipid.

Saponification number. A sample of 0·4463 g oil saponified with alcoholic KOH and back titrated with alcoholic H_2SO_4, was used. The S.N. was found to be 185·6.

Acid number. Titration of 0·03 g of the oil in benzene with potassium alcoholate, with phenolphthalein as indicator, showed that there was a very small but detectable amount of free acid in the oil. The acid number was 0·015.

Iodine number. Toms's bromine method (Toms, 1928) was applied to 0·02 or 0·03 g of oil. Two determinations gave values of 214 and 209. Using Wijs's method (Hawk, Oser, and Summerson, 1953) with 0·2 g of oil, values of 215 and 176 were obtained on different occasions (giving a mean I.V. of 207).

Thiocyanogen number. The technique outlined by Irwin and others (1936) was followed. A value of 111·0 was obtained.

Proportion of saturated acids. Hilditch's modification (1947) of Twitchell's method of estimating the proportion of saturated acids in an oil, depending on the insolubility of the lead soaps of saturated acids in alcohol, was used with a 0·1 g sample of oil. It was found that about 15% of the total fatty acids were saturated.

Unsaponifiable fraction. Diethyl ether extracts of the diluted saponified extract used in the Hilditch–Twitchell procedure indicated that the total amount of unsaponfiable matter in the oil was small; it has not been determined.

Proportion of polyethenoid acids. The lithium salts of the fatty acids were separated from 95% acetone by the technique of Hilditch and Maddison (1942). Two determinations showed that 70 to 75% of the fatty acids in the oil have soluble lithium salts (polyethenoid acids of the C_{20-24} series).

The quantity of coelomic cells

It has been mentioned already that the dry weight of the cells (minus oil) may amount to as much as 75% of that of the remainder of the body; that is, rather less than 40% of the total dry weight (minus oil) of the whole worm. There is also an approximate relationship between the total quantity of cells and the dry weight of the remainder (fig. 8). In the presence of gametes these may be separated from the cells by centrifugation, and in the female the quantity of cells is seen to diminish as the oocytes mature. The cells probably never disappear completely, however, and as far as may be judged from 7 females apparently just ready to spawn in January, there remains between

0·1 and 0·2 g (dry weight, minus oil) of cells in worms of 0·5 to 1·0 g dry body-weight; that is about 18%. From this it may be estimated that 75 − 18 = 57% of the dry weight of the rest of the body is expended annually in the form of oocytes. The basal level of 18% is gradually augmented, as is shown in fig. 8.

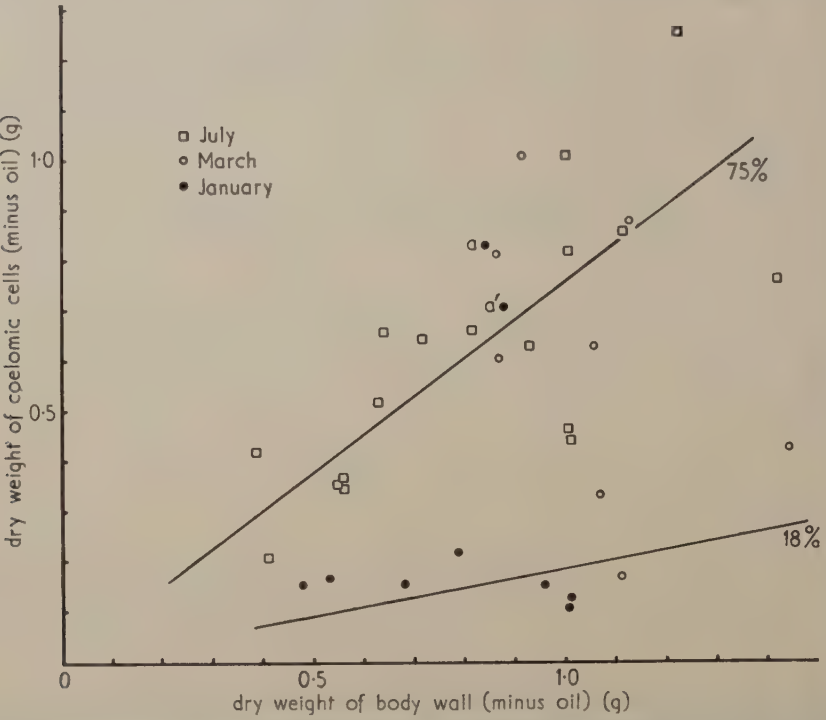


FIG. 8. *S. spallanzanii.* Relationship between the weight of the coelomic cells and the weight of the remainder of the body. Points *a*, *a′* are from non-breeding worms; the quantity of coelomic cells of normally breeding worms in January approximate to 18% of the remainder of the body (lower line); in July, 75% (upper line).

In two instances calculated, the actual expenditure was 50% and 52% respectively. The relevant data for one of these, which was clearly just about to spawn and in which the best separation of the cells and oocytes was achieved, are given in table 2 (see appendix).

The quantity of oil and glycogen in the coelomic cells and oocytes

In order to determine the weight of oil and glycogen, and the total dry weight of the same specimen, the following procedure was adopted. Advantage was taken of the solubility of oils in 2 : 1 mixture of chloroform and methanol in the cold, and of the simultaneous precipitation of glycogen. After extraction of the oil, the residue was dried in a desiccator, weighed, and the glycogen estimated.

The worms were slit open and the coelomic contents flushed out with sea-water. As the development of septa is extensive, it was found best to make two dorso-lateral slits. By joining these by a transverse cut and stripping back the dorsal muscle-bands, the gut could then more easily be removed if required for separate estimation. The tissue (gut or body-wall) was then disintegrated with a Sperry pattern disintegrator. Coelomic cells were placed directly after centrifuging in the solvent mixture. Care had to be exercised when centrifuging: a speed of only 500 rev/min would sometimes rupture the cells and much of the oil would then rise to the surface. The cells were rinsed with sea-water and centrifuged again. Any spermatozoa present separated as an upper layer and were removed for separate determination; oocytes formed a lower layer. When gametes were present repeated centrifugation was necessary to effect complete separation. Extraction and purification followed by weighing of the oil was carried out as recommended by Sperry (Glick, 1955) and as described in a previous paper (Dales, 1958). After extraction of oil, the cells were dried at 100°, cooled in a desiccator, and weighed. This weight (dry weight minus oil) plus the weight of oil, was taken as the 'total dry weight' of the sample. Glycogen was then determined after digesting in 30% KOH and reprecipitating with alcohol, dissolving in hot water to a known volume, hydrolysing a sub-sample with 0·6 N HCl, and estimating the resulting glucose by Hagedorn and Jensen's 1923 method as previously described (Dales, 1957).

The relative quantities of oil and glycogen in the coelomic cells as compared with the rest of the body are presented in table 3. It will be seen, for example, that in March there was 3 times as much oil and twice as much glycogen in the cells as in the remainder of the body.

Fig. 9 presents the result of many determinations of oil and glycogen in the coelomic cells throughout the year expressed as percentages of their total dry weights. There would seem to be relatively more oil in March than in July, and more in January than in March. Also, as the oil increases the glycogen decreases, and there is a statistically significant (negative) correlation between the two components. This effect might be due to (1) the type of food available at different seasons—winter flagellates may have a higher oil content; and/or to (2) the active conversion of glycogen into oil by the cells. In this connexion the proportion of the two types of substance in the oocytes is interesting. In three instances in which a good separation of cells and oocytes was achieved, the oil content was found to represent only 35 to 40 % of the total dry weight, the glycogen content only 3 to 6% (table 4). This small proportion of glycogen is more like that of the coelomic cells in January (when spawning occurs) than in July, when the proportion of glycogen is much higher (fig. 9).

Some calculations based on the determinations given in table 4 reveal facts which may have some bearing on this problem. Percentages are here expressed as dry weight minus oil throughout.

In the oocytes, oil represents about 60% of the dry weight, glycogen 7·3%. For a worm of total dry weight 1·0 g, the oocytes would contain a total of

0·6 g oil and 0·073 g glycogen.

18% of 1·0 g or 0·18 g of cells may be left from the previous spawning (see above); of which, in the cells:

oil = 134% or 0·241 g,
glycogen = 22% or 0·039 g,

which is equivalent to a little less than one-half of the total quantity in the oocytes. The maximum quantity of cells in July is 75% of the dry weight of the remainder of the body or 0·75 g.

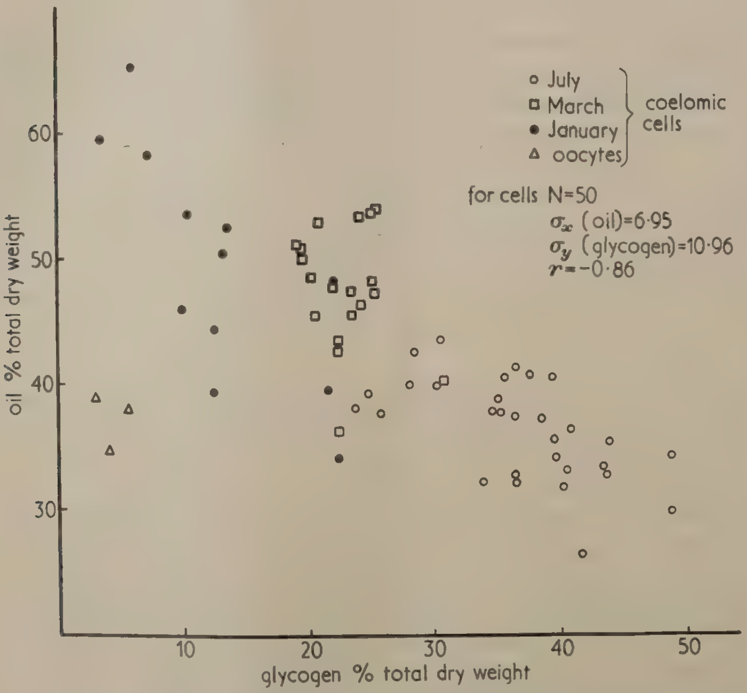


FIG. 9. *S. spallanzanii.* Relationship between the quantity of glycogen and oil in the coelomic cells at different seasons. Each component is expressed as % of the total dry weight of the coelomic cells analysed in each worm.

Total mean oil content, therefore = 57% or 0·428 g approximately;
total mean glycogen content = 58·5% or 0·439 g approximately;
0·439—0·073 = 0·366+0·428 = 0·794 g.

Some glycogen built up during the summer may be converted into oil, for not all the oil present in the summer in the coelomic cells can account for the quantity found in the oocytes in January.

If for purposes of argument it is assumed that *all* the reserves of the cells are made over to the oocytes, then:

$$\begin{array}{rlrl} \text{total oil in oocytes} = & 0{\cdot}6 & \text{total glycogen} = & 0{\cdot}073 \\ \text{cells} = & \underline{0{\cdot}241} & = & \underline{0{\cdot}039} \end{array}$$

$$\underbrace{0{\cdot}841 \qquad + \qquad 0{\cdot}112}_{0{\cdot}953\ \text{g}}$$

and it may be calculated that in July the cells contain:

$$\text{oil} = \underbrace{0{\cdot}428\ \text{g} + \text{glycogen} = 0{\cdot}439}_{0{\cdot}867\ \text{g}}$$

The glycogen stores and anaerobiosis

In view of the large quantity of glycogen in the coelomic cells and the fairly high sea temperatues (24° C) that these worms withstand in summer some worms were kept under anaerobic conditions at this temperature to see if the glycogen in the coelomic cells was depleted. A continuous stream of pure nitrogen was maintained through vessels containing the animals and closed by bunsen valves (Dales, 1958) for 20 to 24 h. There was no significant difference in the glycogen content at the end of the experiment, and it would seem that these particular reserves are not drawn upon under adverse conditions. This agrees with the results obtained with *Owenia* (Dales, 1958).

The Peritoneum and Coelomic Cells in some Other Sabellids

Free coelomic cells may be seen in *S. pavonina* and to a certain extent also in *M. infundibulum*, though the coelomic cavity in *Myxicola* is greatly reduced by the development of the longitudinal muscles. In *S. pavonina* the quantity and size of the coelomic cells is much less than in *S. spallanzanii*, but they appear to increase in quantity towards the time when the oocytes mature in the coelom. In *Branchiomma* the coelomic cells are relatively even smaller. In *E. polymorpha*, *E. vancouveri*, *Schizobranchia insignis*, and *Pseudopotamilla ocellata*, on the contrary, there are few coelomic cells, but in each the peritoneum assumes an orange or brown colour which is due to large quantities of carotenoid, other pigments, and oil. Where these deposits are to be found the peritoneum is proliferated into lobes. It forms a pair of raised ridges near the ventral vessel in *Myxicola*, while in *Eudistylia* and *Schizobranchia* the peritoneum is elaborated into leaf-like villi projecting into the coelom (fig. 11, B). Cells of similar appearance may be found also around the oblique muscles in *Schizobranchia* (fig. 10). In all these instances this elaborated peritoneum is not directly related to the blood-vascular system. In *Eudistylia* and *Schizobranchia*, for example, where blind-ending vessels project into the coelom on the dorsal side, the peritoneal villi are clearly separate from the cells marginal to them (fig. 10, B). The chloragogen cells, which are always related to the blood-vessels, are quite different in appearance and are quite distinct (figs. 5, E, F; 11, A). All the specimens belonging to these species which were examined in June, July, and August had a villous peritoneum; whether this is so also at other seasons is not known. In *Pseudopotamilla* one specimen with ripe spermatozoa was found in August. This had many cells free in the coelom. Thus at this time the peritoneum may proliferate cells into the coelomic fluid. *Eudistylia* and *Schizobranchia* have very friable peritoneal villi, and cells may easily be brushed off. In *E. polymorpha* small pieces of broken villi or masses of cells were often found free in the coelom. Some of these were dark brown, the brownish granules staining slightly but not readily with Sudan black B. Possibly they represent chromolipids derived from phospholipids.

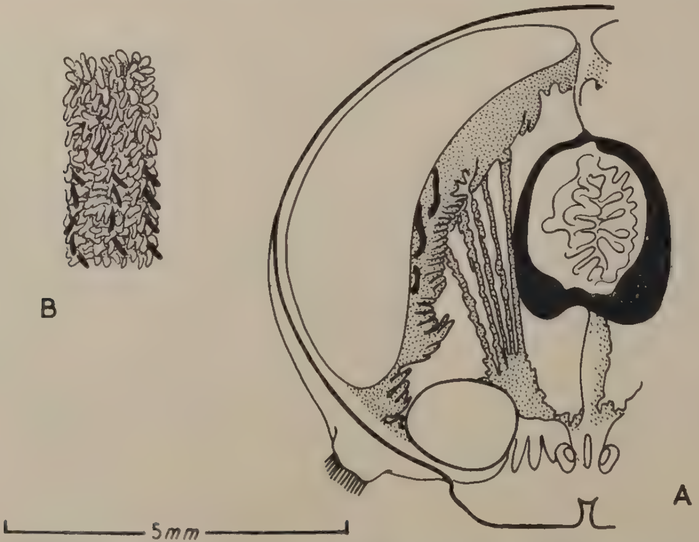


FIG. 10. *Schizobranchia insignis*, showing the villous peritoneum. A, illustrating the extent of the elaborated peritoneum (August); cells loaded with oil and carotenoid extend up the oblique muscles. B, surface view of a transverse strip of peritoneum taken from the dorsal side, showing the villi and blind-ending coelomic blood-vessels between.

Smears were made for histochemical examination and frozen sections made of tissue fixed in formaldehyde / calcium. Tissue fixed in Bouin was also subjected to routine staining after paraffin embedding and sectioning.

Cain's (1947) Nile blue and Menschik's (1953) Nile blue showed that phospholipid was present in all the globules, which coloured heavily with Casselman's acetylated Sudan black B in propylene glycol. Frozen sections coloured with Sudan black B, or with oil red O or Fettrot 7 B (Gurr) in

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FIG. 11 (plate). A, *S. pavonina*. T.S. showing the ventral vessel and a pair of segmental vessels above the nerve-cord, surrounded by deeply pigmented chloragogen cells (*chlc*) with free coelomic cells (*cc*) in the coelom. (Azan.)

B, *Eudistylia vancouveri*. T.S. through the peritoneal villi. The large empty spaces (*o*) are left by the dissolved oil. (Azan.)

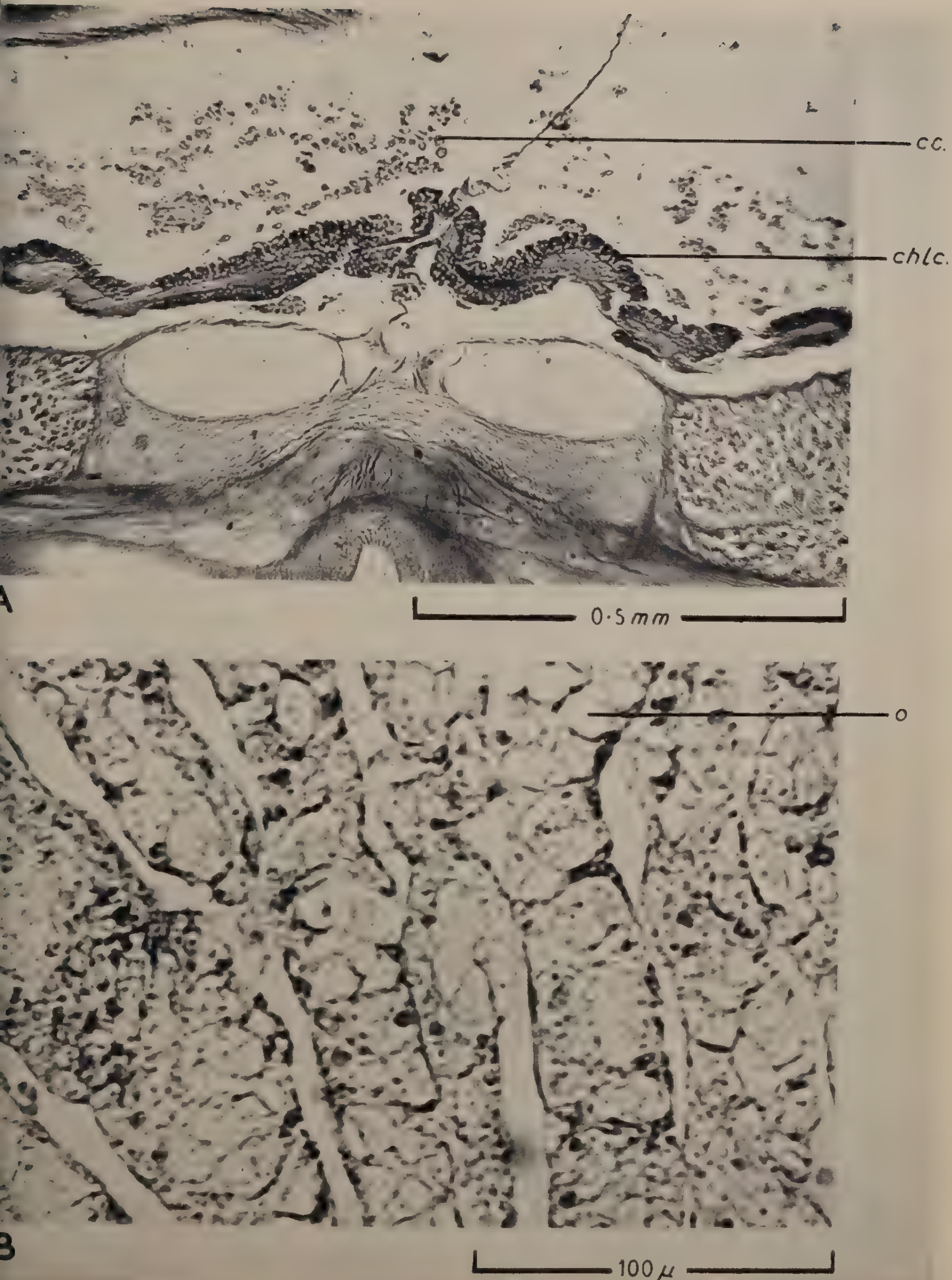

Fig. 11

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olyethylene glycol (Zugibe and others, 1959) demonstrated the very large oil ontent of the peritoneum as compared with other tissues. Six quantitative eterminations of the oil content gave a mean value of 60% of the dry weight minus oil), a value very similar to that of *Sabella* cells. Casselman's technique ave the most delicate localization of the oil in the frozen sections. Not all f the lipid appeared to be extracted with hot acetone, and this greater resisance may be due to storage of the smears for rather a long time (several weeks) n formaldehyde / calcium before colouring (Baker, 1945). Hot pyridine and he mixture of chloroform and methanol, refluxed for 24 h, were used for ontrols.

CONCLUSIONS

There seems to be little doubt that the coelomic cells of *S. spallanzanii* are rganized as storage centres for the maturation of the gametes, and that their eserves of oil and glycogen are not normally drawn upon for other purposes r at times of inanition. The quantitative estimations show that most if not all f the reserves they contain are made over to the oocytes. The elaboration of he peritoneum in *Eudistylia* and *Schizobranchia*, for example, suggests that he free coelomic cells of *Sabella* are derived from the peritoneum. The enarged peritoneal ridges near the ventral vessel in *Myxicola* as well as the resence of free cells also suggests this. Smears of cells from both fully grown nd very small *S. spallanzanii* stained with acetic orcein failed to show any ivision of the cells in the coelom, and they may well be recruited from the eritoneum as small, actively phagocytic cells. The size of these is clearly ependent on the absolute size of the worm, the optimal coelomic cell size eing attained at an early stage in the life history (fig. 1). The length of life of coelomic cell is not known, but it seems likely that the cell when replete vith reserves eventually ruptures, liberating its inclusions into the coelomic luid to be taken up by younger cells or by the gametes. Apparently old cells in vhich the cell membrane has disappeared, or even free groups of oil-globules nay often be seen in apparently normal and healthy worms. The smallest ells naturally take up the smallest particles first, so that they are often loaded vith granules of carotenoid and little oil. This distinction between the arotenoid and oil is noteworthy, for these pigments are strongly oil-soluble. n *Schizobranchia* the carotenoids are dissolved in the oil occurring in the eritoneal cells. In *Myxicola* most of the carotenoid stored in the body is, owever, deposited in the integument. This may be correlated with the very estricted coelomic space and relatively poor development of the peritoneal issue and free coelomic cells, but might equally well be directly related to the act that, unlike the opaque tubes of most sabellids, the transparent gelatinous ubes, though buried, may allow penetration of some light or short wave adiation from above.

The phagocytic activity of the coelomic cells leads them to pick up other ubstances, probably including excretory end products, so that older worms vith a darkening body commonly have coelomic cells with a similar dark colour. Claparède (1868), for example, noticed in *Sabella spallanzanii* that green granules were present in the cells (which he emphasized were not detached chloragogen cells), the older cells often becoming little more than sacs of green pigment. This was confirmed by Romieu (1923). This colour may be due partly to resorbed oocytes or to the flavin-like pigment. Some female worms kept in the aquarium at Plymouth did not spawn successfully in January, and the coelomic cells had, by March, assumed a muddy green colour probably owing to pigments resorbed from the oocytes.

The histochemical work suggests that there is no apparent morphological differentiation of the different fat fractions within the cells and that all the various components form a mixture within the large globules. These lipid globules may therefore be said to consist of a mixture of glycerides with about 18% in the form of phospholipid, and with a very small amount of free fatty acid, and a small proportion of unsaponifiable matter. Very little is known about the composition of fats in invertebrates, but the general composition of the oil in the coelomic cells of *S. spallanzanii* is like that of other marine animal fats: that is to say, the oil has a low melting-point, is highly unsaturated with a large proportion of C_{18-24} acids, and there is a small fraction of unsaponfiable matter. The high I.N. (207) and T.N. (111) together with a S.N. of 185·6 implies a high proportion of unsaturated acids. Further, since the Twitchell separation showed that 15% of the fatty acids were saturated, the remaining 85% must be of a highly unsaturated nature to account for the high I.N. and T.N. of the crude oil. Cod liver oil contains 15% of saturated acids and 35 to 40% of highly unsaturated polyethenoid acids of the C_{20-22} series (Collin and others, 1934). These workers found C_{20-24} acids in phytoplankton; 16% being C_{20} acids in *Phaeodactylum tricornutum* Bohlin according to Lovern (1936), and 50% C_{22} and C_{24} acids in *M. edulis* (Lovern, 1938), which also contained 20% of its phospholipids containing acids of high molecular weight (? C_{28}). Like *Mytilus*, *S. spallanzanii* is a filter-feeder and may be deriving its oil from similar sources. The value of 70 to 75% of polyethenoid acids of the C_{20+} series is certainly higher than has been found before, but does account for the high I.N.

There remains the interesting problem of the actual way in which the food substances are conveyed to the free coelomic cells in *Sabella*. The fact that they are liberated at an early stage into the coelom, and that the peritoneal villi of *Eudistylia* or *Schizobranchia*, for example, have no direct connexion with the blood-system, suggest that the reserves are taken up from the coelomic fluid. It is planned to investigate this further.

The carotenoid accumulated by the cells or peritoneal villi is presumably derived from the food. This is also being investigated further, but preliminary feeding experiments with uni-algal cultures of known carotenoid content indicate that some of these compounds but not others are retained or converted by the worms, and that the deposits in the coelomic cells do not have a rapid turnover. The presence of a compound flavin in the cells as well as the oocytes, and of the same carotenoids in both, also suggests their relationship.

The similar proportions of glycogen and oil at the time of spawning again suggests that the main function of the cells is in the maturation of the oocytes. In males the carotenoids disappear as the coelomic cells diminish.

The coelomic cells of *Sabella spallanzanii* were observed by Claparède (1868); Kollman (1908) drew attention to the quantity of albuminoid granules present, and his observations were confirmed by Romieu (1923). Romieu believed that the coelomic cells when replete with oil (éléocytes) were derived from the smaller active phagocytes. This was questioned by Liebman (1946), who made a distinction between the eleocytes (trephocytes) and the phagocytic cells (lymphoidocytes) and maintained that one did not give rise to the other. The evidence presented here shows that even virtually replete cells may be phagocytic. Measurement of cells in any worm also shows that cells which Liebman would have described as lymphoidocytes and trephocytes form a normal size-frequency curve, and this is not in accord with his view but confirms Romieu's.

The fact that the coelomic or villous peritoneal cells are quite distinct from what are usually termed chloragogen cells in polychaetes (Dales, 1957) is also of some interest. While the coelomic cells are concerned with the uptake and accumulation of food reserves, their phagocytic powers will, of course, lead to their accumulating any foreign or excretory particles in the coelomic fluid. Urich's recent work (1957, 1958) on the chloragogen cells of earthworms suggests a close similarity in function, as he finds that they are also concerned with uptake and storage of oil. The chloragogen cells of the earthworm as well as the cells of the botryoidal tissue in leeches (Bradbury, 1959), however, are associated with the blood-vessels; the peritoneal and coelomic cells of sabellids only with the coelomic fluid. The chloragogen cells of the earthworms appear to store excretory substances as well (van Gansen, 1956, 1957, 1958; van Gansen and Vandermeerssche, 1958), and their functions are clearly complex. What are termed chloragogen cells in polychaetes may also be concerned with the accumulation of excretory compounds, as they tend to increase in quantity and to darken with age, but are in addition apparently haematopoietic (Kennedy and Dales, 1958) and in contact with the blood-vessels. The distinction of the chloragogen cells from the peritoneal and coelomic cells is well seen in *Myxicola* and *Sabella* (figs. 5, E, F; 11, A).

The chemical constitution, other than ionic content, of the coelomic fluid in polychaetes is virtually unknown; it is planned to investigate this in the near future in view of its possible participation in the transport or storage of metabolic products.

An opportunity to study *S. spallanzanii* at the Stazione Zoologica, Napoli, was made possible through the generosity of the Royal Society by a grant from the Browne Fund in 1958; a further grant in 1959 was made towards the expenses incurred in the United States. I am also much indebted to the Directors and Staffs of the marine laboratories at Naples, Santander, Monaco, and Plymouth, the Hopkins Marine Station (Stanford University), and the Friday

Harbor Laboratories of the University of Washington for their kindness and co-operation. I also wish to thank Mr. Rimmon C. Fay of the Pacific Bio-Marine Supply Company, Los Angeles, for collecting *E. polymorpha*, and Mr. Angelo Sessa for help with photography when at Naples.

APPENDIX

Table 1

Total carotenoid in the coelomic cells as compared with the body-wall (in the same individuals)

Total $CHCl_3$:MeOH extract made up to 50 ml or multiple; absorption measured at 460 mμ (July, 1958)

Dry-weight body-wall minus oil	*Beckman reading*	*Dry-weight cells minus oil*	*Beckman reading*
(g)		(g)	
0·2317	0·167	0·0973	0·295
0·5369	0·492	0·1301	0·649
0·2933	0·252	0·0355	0·300
0·3180	0·350	0·1388	0·520
0·5951	0·298	0·1733	0·443
0·4335	0·104	0·1954	0·323

Table 2

Oil content and proportion (dry weight) of the coelomic cells and oocytes as compared with the rest of the body (worm 6). Weights in grams

Body-wall+gut			*Coelomic cells*			*Oocytes*		
Dry wt. minus oil	*Oil*	*'Total dry wt.'*	*Dry wt. minus oil*	*Oil*	*'Total dry wt.'*	*Dry wt. minus oil*	*Oil*	*'Total dry wt.'*
1·0648	0·1623	1·2271	0·1015	0·1891	0·2906	0·4560	0·2972	0·7532

Total dry-weight of body-wall+gut+coelomic cells = 1·5177 g.
Total dry weight of oocytes = 0·7532 g or approx. 50%.

Table 3

Glycogen and oil content of different parts of the body (mean of 6; March 1958). Weights in grams

	Dry wt. minus oil	*Total glycogen*	*Total oil*	*Total dry wt. (plus oil)*	*% glycogen (dry wt. minus oil)*	*% oil (dry wt. minus oil)*
Body-wall . .	0·8101	0·0568	0·0748	0·8849	7·0	9·2
Gut . . .	0·1425	0·0031	0·0226	0·1651	2·2	15·8
Coelomic cells .	0·3389	0·1335	0·2787	0·6176	38·8	81·5

TABLE 4

Comparison of the dry weight, oil, and glycogen content of the coelomic cells and the oocytes (worms 2, 6, and 7). Weights in grams

Worm number	*Coelomic cells*	*Oocytes*	*Total quantity in coelom*
	dry wt. minus oil		
2	0·1535	0·3692	= 0·5227
6	0·1015	0·4560	= 0·5575
7	0·2435	0·1852	= 0·4287
	oil		
2	0·1554	0·2021	= 0·3575
6	0·1891	0·2972	= 0·4863
7	0·2805	0·1084	= 0·3889
	glycogen		
2	0·0413	0·0275	= 0·0688
6	0·0175	0·0225	= 0·0400
7	0·0538	0·0175	= 0·0713

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The Development of the Malpighian Tubules in *Dysdercus koenigi* (Hemiptera, Pyrrhocoridae)

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With two plates (figs. 5 and 7)

SUMMARY

Dysdercus koenigi has two pairs of Malpighian tubules, each pair arising from a vesicle and forming a closed loop. The vesicles are connected dorsally, and the tubules are differentiated into proximal and distal regions with refractive granules in the latter and brush border in both.

In winters at Allahabad the embryonic period lasts about a fortnight, and on the 9th day, 4 small buds arise apparently from the anterior end of the proctodaeum. These grow rapidly and become tubular; their tips fuse, but the lumina remain separate. At this stage the met-enteric membrane (Henson, 1946*a*) stretches over the anterior end of the proctodaeum and an interstitial ring is differentiated, so that the zone of the origin of the tubules lies anteriorly to it and posteriorly to the met-enteric membrane. Meanwhile the basal part of each tubule swells and the two swellings of each side fuse, thus forming a vesicle on each side. A dorsal outgrowth from each vesicle meets its fellow of the opposite side. The tubules continue to elongate, at the same time becoming narrower, and ultimately only 3 cells can be seen in a cross-section. The tubules grow in 3 phases—(1) active mitosis, (2) of cell multiplication and cell rearrangement, and (3) of cell rearrangement and cell enlargement. Finally, the tips of each pair fuse completely and their lumina become continuous.

In the newly hatched first nymph the tubules are uniform throughout, but in the later part of this stage they become differentiated into proximal and distal regions and the striated or brush borders appear. Thus the embryonic development continues for some time in the first nymph. Once this condition has been reached, cyclical changes involving increase in the diameter of the tubules, in the size of the nuclei, and in the number of cells in the proximal region occur in each nymphal stage, but before each ecdysis the diameter decreases slightly. Thus the adult condition is reached in 5 distinct steps, corresponding to the 5 instars.

Henson's view that Malpighian tubules are endodermal is considered plausible, but his view regarding the primitive number of tubules is not accepted.

INTRODUCTION

AMONG the more important works on the embryonic development of the Malpighian tubules of insects are those of Wheeler (1889) on *Blatta germanica* and *Doryphora decemlineata*, Carrière (1890) on *Chalicodoma*, Nelson (1915) on *Apis*, Samson (1908) on *Heterogenea limacodes* and Ikeda (1913) and Ito (1921) on *Bombyx mori*. Later, Henson in a series of papers (1937, 1944, 1946) described the development of Malpighian tubules in *Pieris brassicae*, *Blatta orientalis*, and *Forficula auricularia*. Contradicting the earlier opinion, he stated that they are endodermal in origin. Drummond (1936), Thomas (1936), Paterson (1936), Mellanby (1937), and Butt (1949)

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referred to the development of the tubules in the course of their embryological works on *Ephestia*, *Carausius*, *Corynodes*, *Rhodnius*, and *Oncopeltus* respectively. Of these, except Butt, who is uncertain, all regard the tubules as ectodermal. Recently Savage (1956) has described the development of the tubules in *Schistocerca gregaria* and supported Henson.

It is thus clear that we are still far from certain regarding the fundamental nature of the tubules; and in the Hemiptera there has been no exhaustive study so far. In the present paper, therefore, an attempt has been made to furnish observations on the embryonic and post-embryonic development in a typical Heteropteran, *Dysdercus koenigi* (Fabr.) and to compare the results with such facts as we possess about other insects.

MATERIAL AND TECHNIQUE

Specimens of *D. koenigi* were reared under normal laboratory conditions during the months of December and January, when the outside temperature ranged from 6·5° C to 27° C. The eggs were removed soon after they were laid, kept separately, and fixed at regular intervals. Among the fixatives used, cold Carnoy's gave the best results. To ensure complete penetration of the fixative, the shell was carefully removed with fine needles after the egg had been kept in the fixative for some time. Transverse and longitudinal sections were cut at 5 to 8 μ; they were stained in Delafield's haematoxylin and sometimes counterstained with eosin.

OBSERVATIONS

The adult

D. koenigi has two pairs of Malpighian tubules, each pair arising from a small, laterally placed vesicle and forming a closed loop with the distal ends joined with each other (fig. 1). The vesicles are connected with each other dorsally, and each vesicle opens into the lower part of the mid-gut. The tubules are differentiated clearly into distal and proximal regions, with abundant granules in the cells of the former. Both the regions show an inner brush border.

Embryonic development

In December and January the embryonic development of *Dysdercus* is completed in about 15 days. When the embryo is about 9 days old, the Malpighian tubules make their first appearance as 4 minute buds (fig. 5, A) from the lateral and ventro-lateral sides of the anterior region of the hind-gut. This may be designated as the embryonic zone, as active cell-division takes place in it for some time (figs. 3, A; 5, B). The buds consist of 17 to 20 columnar cells measuring about 14 μ tall and arranged in a single layer (fig. 2, A). The cell-walls are not distinct. The lumen of the bud is only 7 μ wide. There is no indication of a striated border on the inner margin of the cells. The nuclei, which are large and basiphil, measure 5 by 4 μ. Many mitotic figures are seen in longitudinal sections; this shows that active longitudinal cell-division is in progress.

In about an hour the length of the bud increases to about 64 μ, but its diameter and the number of cells in a cross-section remain nearly the same.

In an embryo 9 days, 8 h old, each tubule is about 90 μ long and about 36 μ in diameter. The tips of the two tubules of each side now join, although their lumina remain separate (fig. 5, B). The tubules retain the histological character described in the earlier stage. Longitudinally arranged mitotic figures are still observed; this is correlated with increase in the length of the tubules. Sections at this stage show that the anterior walls of the tubules are continuous with the wall of the mid-gut and the posterior wall with the wall of the hind-gut. A thin membrane stretches across the anterior end of the proctodaeum (figs. 3, B; 5, F). It marks the posterior end of the mid-gut and is called the met-enteric membrane (Henson, 1946*a*). In an embryo 9 days, 6 h old, the region of the union of the posterior walls of the tubules and proctodaeum differentiates in a region distinct histologically from the mid-gut as well as from the proctodaeum: this is called the posterior interstitial ring (figs. 4; 5, H). Later, it develops into the proctodaeal valve. Mitotic division occurs in this ring. It may be noted that the tubules now lie anteriorly to this ring.

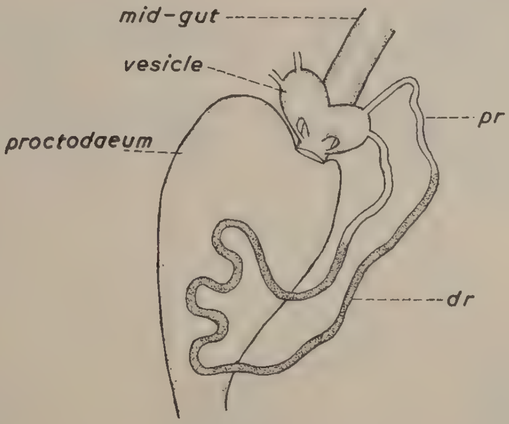

FIG. 1. Diagrammatic representation of the posterior part of the alimentary canal of adult *Dysdercus koenigi*, showing the arrangement of the Malpighian tubules. *dr*, distal region of the Malpighian tubule; *pr*, proximal region of the Malpighian tubule.

In an embryo 9 days, 12 h old, the basal part of each tubule has swollen on account of active cell-division (figs. 3, C; 5, G), and subsequently, on account of greater growth in the dorsal and ventral walls of the swellings of the upper and lower tubules of the same pair, they have come close and finally fused, thus forming a single vesicle from which both the tubules of a pair now appear to arise (fig. 5, B, C). Later an outgrowth arises towards the median line from the dorsal side of each vesicle. The two outgrowths meet each other. The met-enteric membrane cannot be identified at this stage and the cells forming the vesicle resemble those of the rest of the Malpighian tubule. The tubules are straight, about 108 μ long and 28 μ in diameter, with 12 to 15 cells in a cross-section. Mitotic division still continues (fig. 2, B). The decrease in the number of cells in cross-sections, without cessation of mitosis, shows that the processes of cell arrangement and cell production are now progressing simultaneously.

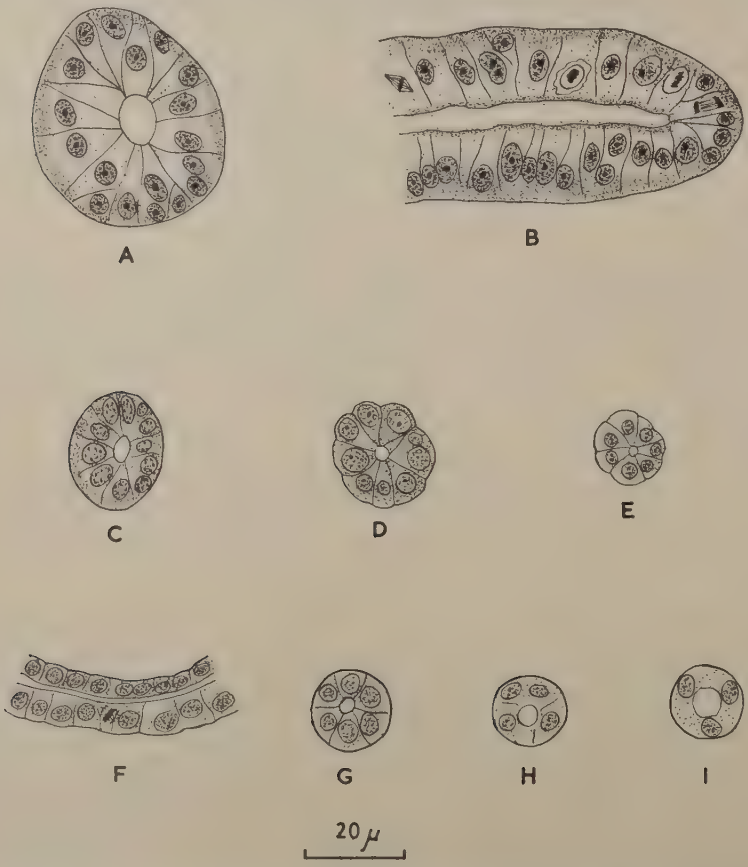

FIG. 2. T.S. and L.S. of the Malpighian tubules of embryos of different ages. A, T.S. at 9 days, 3 h stage. B, L.S. of apical part at 9 days, 12 h stage. C, T.S. at 9 days, 16 h stage. D, T.S. at 10 days, 12 h stage. E, T.S. at 11 days, 12 h stage. F, L.S. at 11 days, 20 h stage. G, T.S. at 12 days, 20 h stage. H, T.S. at 13 days, 12 h stage. I, T.S. at 14 days, 3 h stage.

In an embryo 9 days, 6 h old, the length of each tubule has increased to about 136 μ and it has become slightly twisted in the form of an inverted S. Its diameter has decreased further to about 25 μ and there are only 10 to 12 cells in a cross-section (fig. 2, C). The vesicles have become further enlarged and their outer walls have become covered by thin muscles.

By the time the embryo is 10 days, 10 h old, each tubule measures about 256 μ long and about 19 to 22 μ in diameter. It is now coiled to form a few loops. The lumina of the two tubules of a pair are still separate at their tips.

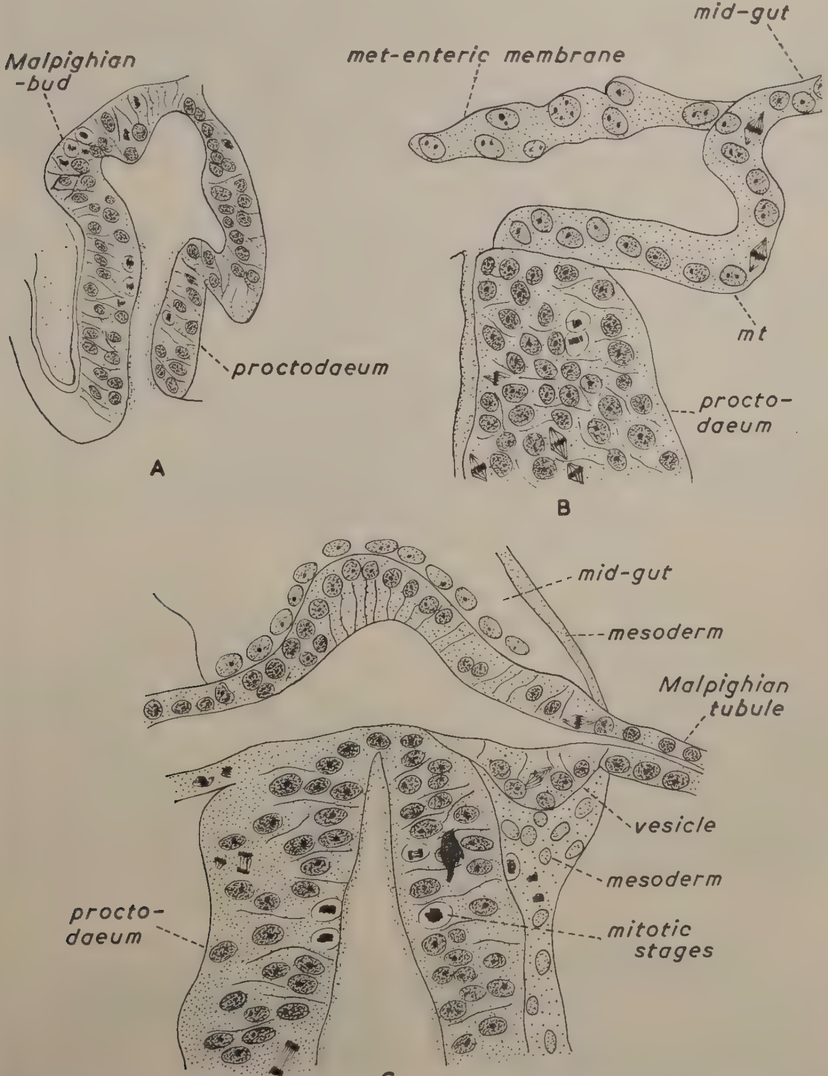

FIG. 3. L.S. of the proctodaeum of different stages. A, Malpighian buds at 9 days, 4 h stage. B, met-enteric membrane at 9 days, 8 h stage. C, formation of vesicle at 9 days, 12 h stage.

In an embryo 10 days, 12 h old, the tubules have 8 to 10 cells in a cross-section (fig. 2, D). Mitotic figures are still seen and the vesicles are better differentiated.

In an embryo 11 days, 12 h old, the length of the tubules has increased to about 0·8 mm and the diameter has decreased to 14 μ. Only 6 to 8 cells are seen in cross-sections (fig. 2, E). There is no other change in the histology o the tubules or the vesicles.

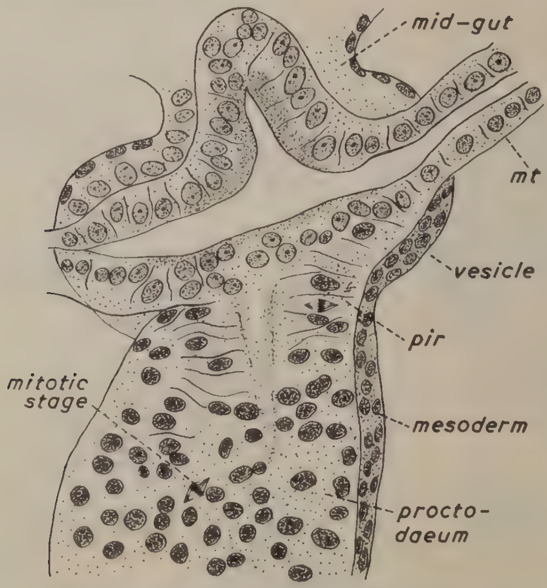

FIG. 4. L.S. of the proctodaeum at the 9 days, 12 h stage, showing the posterior interstitial ring. *mt*, Malpighian tubule; *pir*, posterior interstitial ring.

In an embryo 12 days old, the two tubules of a pair have fused completely and their lumina have become continuous, thus forming a complete loop (fig. 5, D), about 1·92 mm long. The diameter is reduced to about 13 μ and only 6 cells are seen in cross-sections. Cell production now ceases. The striated border has not developed yet. Therefore the tubules are now anatomically similar to, but histologically dissimilar from, those of the adult.

In an embryo 13 days, 12 h old, each loop of the Malpighian tubules measures about 3·2 mm long and 13 μ in diameter, and there are only 3 or 4 cells in cross-sections (fig. 2, H).

FIG. 5 (plate). A to D, photomicrographs of whole mounts at different stages.

A, Malpighian buds at 9 days, 3 h stage (under phase contrast).

B, Malpighian tubules and proctodaeum at 9 days, 16 h stage, showing vesicles and the fusion of tubules at the distal ends.

C, origin of the tubules from the vesicles at the same stage.

D, Malpighian tubules at the 12 days stage, showing complete fusion at the distal ends.

E to H, photomicrographs of longitudinal sections of the proctodaeum.

E, at 9 days, 4 h stage, showing the Malpighian buds.

F, part of L.S. at 9 days, 8 h stage, showing the met-enteric membrane.

G, at 9 days, 12 h stage, showing the formation of a vesicle.

H, part of L.S. at 9 days, 16 h stage, showing the posterior interstitial ring.

lp, loop; *m*, mitotic stage; *mb*, Malpighian bud; *mem*, met-enteric membrane; *mt*, Malpighian tubule; *pir*, posterior interstitial ring; *v*, vesicle.

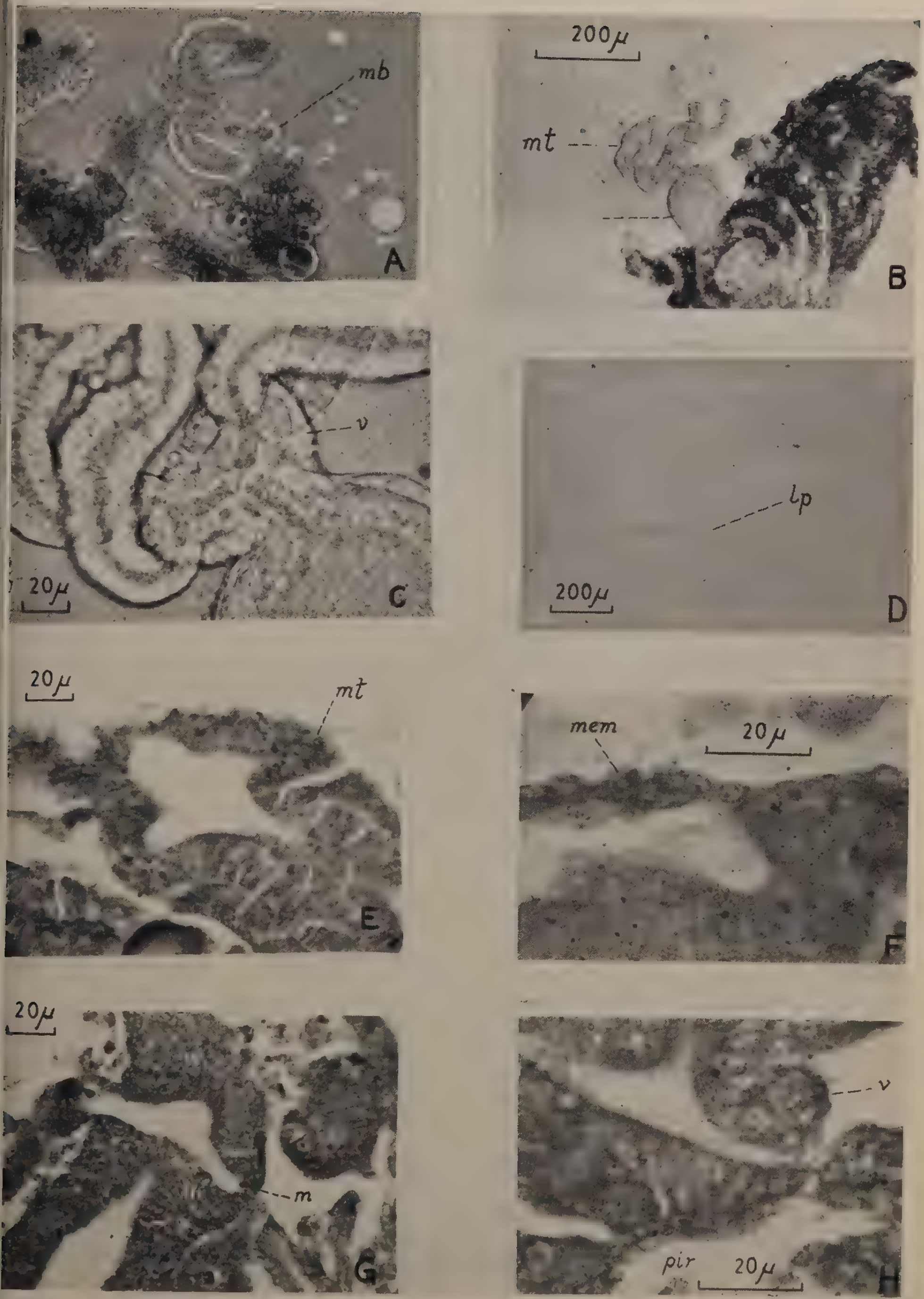

Fig. 5

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By the time the embryo is 14 days, 3 h old, the loops become about 3·52 mm long and about 13 μ in diameter, with only 3 cells in cross-sections (fig. 2, I).

In an embryo 14 days, 17 h old, i.e. a few hours before hatching, the tubules have all the characteristics of the tubules of the adult apart from the differentiation of the striated border and of the proximal and distal regions.

It will be seen that the entire period of embryonic development can be divided into 3 phases. In the first phase of short duration, the tubules appear and increase in length by active mitosis; in the second phase, both cell production and cell rearrangement occur, and increase in the length of the tubule is accompanied by reduction in diameter; in the third phase, cell production ceases and cell rearrangement and cell enlargement take place so that while the diameter of the tubules remains constant, their length increases and at the same time the number of cells in a cross-section decreases.

During the embryonic development of the Malpighian tubules the nuclei show a steady reduction in size. The nuclear size in the different stages, along with the length, diameter, number of cells in cross-sections of the tubules, &c., are given in table I (see appendix).

Post-embryonic changes

In the first nymphal stage the Malpighian tubules, in the newly hatched condition, are devoid of the striated border (fig. 7, A) and of differentiation into proximal and distal regions (fig. 6, A, B). There are 3 cells in a cross-section, each about 5 μ in height, and the lumen is only about 6 μ in diameter. The oval, basophil nuclei measure about 4 by 3 μ. In 2 days, 12 h the diameter and length have increased to about 25 μ and 4·48 mm respectively. As the nymph advances further in age, the size of the cells and the length of the tubules increase further, and at the same time the tubule is differentiated into proximal and distal regions on account of the deposition of certain refractive granules in the cells of the latter.

Meanwhile, the inner margin of the cells becomes frilled, and in a nymph of 3 days it is broken to form the brush border.

In the fully developed first nymph, the diameter of the tubules has increased to 25 μ; but shortly before ecdysis, it is again reduced to about 22 μ. The size of the nuclei remains more or less the same throughout the life of the first nymph.

In the second nymphal stage for some time after the ecdysis, the length and diameter of the Malpighian tubules remain the same as at the time of the ecdysis, but later they increase rapidly to a maximum of 7·36 mm and 40 μ respectively. Both the length and the diameter of the tubules will now remain stationary until before the next ecdysis. The diameter of the nuclei also increases from 5 μ to 6 μ in 2 days, and this diameter persists for the rest of this nymphal stage.

In the I day, 12 h second instar, the differentiation between the proximal and distal regions is more distinct (fig. 7, B). The latter region, besides being

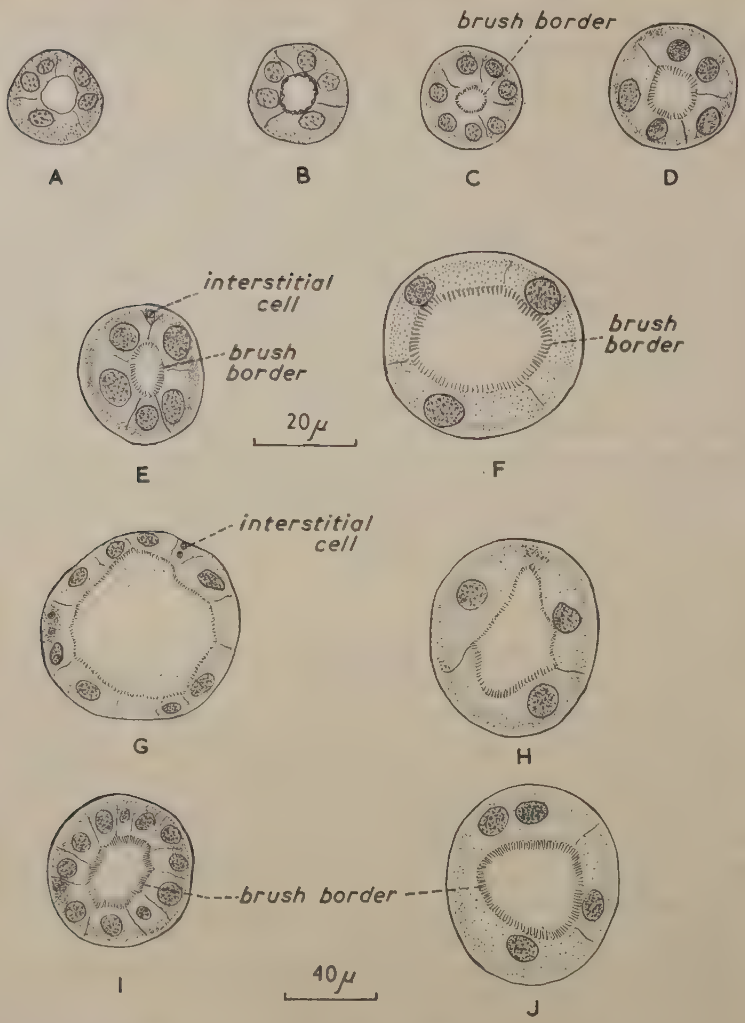

FIG. 6. T.S. of the Malpighian tubules of different stage nymphs. A, of 12 h old first nymph. B, of 3 days old first nymph. C, of proximal region of second nymph. D, of distal region of second nymph. E, of proximal region of third nymph. F, of distal region of third nymph. G, of proximal region of fourth nymph 4 h before the next moult. H, of distal region of fourth nymph 4 h before next moult. I, of proximal region of fifth nymph. J, of distal region of fifth nymph.

packed with granules in the cytoplasm, is now wider (about 24 μ), has 3 cells in cross-section (fig. 6, D), each about 8 μ tall and containing nuclei with a diameter of about 5 μ. The proximal region is narrower (about 18 μ in diameter), and has 7 cells in cross-section, each only about 6 μ tall (fig. 6, C). A brush border is seen in both the regions.

In the third nymphal stage and subsequent instars, there is no marked histological change in the tubules but the distal region is packed with more

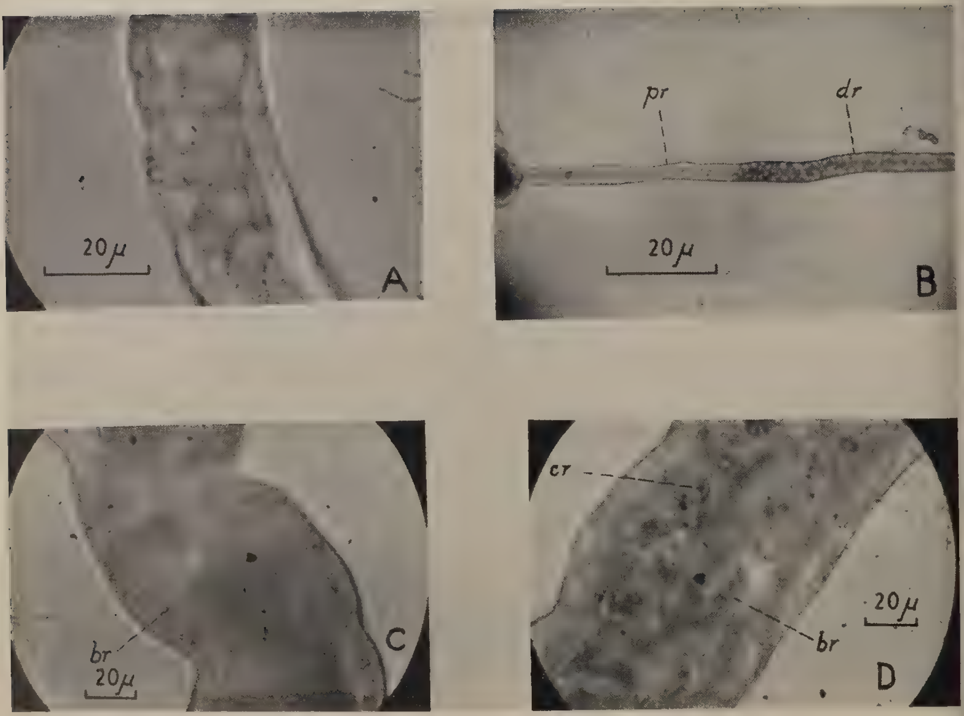

Fig. 7

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anules, and in the proximal region 5 to 8 cells, and a few interstitial cells, are en in cross-section. Mitotic divisions and the replacement of main cells interstitial cells were, however, not observed. The diameter of the distal rt gradually increases and finally reaches a maximum of 61 μ in the 6 days, h nymph. The lumen increases proportionately and the oval nuclei which easured 8 μ by 5 μ become spherical and have a diameter of 11 μ. The ameter of the proximal part increases likewise and the spherical nuclei easure 6 μ in diameter (fig. 6, E, F). During this stage the length of each op increases to approximately 11·2 mm.

In the fourth nymphal stage the length of each Malpighian loop increases to out 15 mm, its maximum diameter to about 83 μ, and the diameter of the clei to 13 μ. In the distal region there are still 3 cells in a cross-section with merous and larger granules in the cytoplasm (fig. 6, H), whereas in the oximal region there are 8 to 12 cells in a cross-section (fig. 6, G). Interstitial lls are present in the proximal region and brush border is seen clearly in th the regions (fig. 7, C, D).

In the fifth nymphal stage, which is much longer, considerable increase in e length and diameter of the tubules is brought about by enlargement of lls. Each loop reaches a length of about 30 mm and the diameter of the bule, which was 72 μ after the last ecdysis in the distal region, reaches a aximum of about 101 μ. The spherical nuclei again become oval and have a e of 22 μ by 14 μ. The distal region has only 3 cells in a cross-section; these 14 μ tall and have a lumen about 40 μ wide. The cells are stuffed with ge refractive granules (fig. 6, J). The proximal region has 8 to 13 cells in oss-section, the cells being about 16 μ tall; the lumen is about 22 μ wide g. 6, I). The cytoplasm is dense and has only a few granules. Interstitial lls are seen here and there.

The increase in the length and maximum diameter of the Malpighian bules in the different stages of the 5 nymphal instars are shown in tables and 3 (see appendix).

DISCUSSION

Wheeler (1889) stated that the Malpighian tubules of *Blatta* and *Doryphora* se from the proctodaeum and are thus ectodermal, and Carrière (1890) d Nelson (1915) reported that in *Chalicodoma* and *Apis* respectively they se from the ectoderm before the proctodaeum is formed. But Tirelli (1929) owed that these tubules arise from an undifferentiated zone between the d-gut and mid-gut, and in 1932 Henson postulated that the proctodaeal

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IG. 7 (plate), Photomicrographs of parts of Malpighian tubules at different stages.
, in the first nymph, showing absence of striated border.
, in the second nymph, showing two regions of a tubule.
, in the distal region of the fourth nymph, showing brush border.
, in the proximal region of the fourth nymph, showing brush border and crystals.
r, brush border; *cr*, crystal; *dr*, distal region of Malpighian tubule; *pr*, proximal region of lpighian tubule.

invagination of insects, like the blastoporal lip of *Peripatus*, is a comple structure. In *Pieris brassicae*, he stated that the posterior interstitial ring an the proctodaeum behind it are ectodermal, while the tissue anterior to th former is endodermal, and so the Malpighian tubules arising from the forme are also endodermal. In *Blatta* he (1944) showed that the membrane coverin the anterior end of the proctodaeum represents the junction of mid- and hind gut and therefore the Malpighian tubules, arising anteriorly from this zone are endodermal. He also homologized the embryonic zone with the posterio interstitial ring of higher Holometabola and labelled the region lying betwee the mesenteron proper and the hind-gut as the met-enteron and the membran covering the hind-gut as the met-enteric membrane.

Recently Savage (1956) has supported the idea of endodermal origin o Malpighian tubules in *Schistocerca*, but Drummond (1936) in Lepidoptera Thomas (1936) in Orthoptera, Paterson (1936) in Coleoptera, and Mellanb in Hemiptera had all described an ectodermal origin. Drummond and Mellanby found that the Malpighian tubules arise from the anterior end of th proctodaeum; Thomas regarded the partition membrane between the mid-gu and hind-gut itself to be ectodermal, and Paterson did not observe the interstitial ring and so regarded the entire proctodaeum as ectodermal. Butt (1949 described the development in the bug *Oncopeltus*, which is similar to Mellanby's, but he considered Henson's view possible.

The present study does not provide sufficient material to permit a detaile evaluation of Henson's views, but it may be mentioned that we did observ in the proctodaeum of *Dysdercus* a specially active zone from which th Malpighian tubules arise, and a region posterior to this zone and behin the Malpighian tubules is later differentiated into a ring which is similar to th interstitial ring of *Vanessa* and the embryonic zone of *Blatta*, although it doe not bud off secondary tubules. Besides, the met-enteric membrane covering th proctodaeum anteriorly and the Malpighian tubules are histologically simila to the mid-gut. Therefore Henson's views regarding the endodermal origi of Malpighian tubules appear highly probable.

As regards the sequence of the division, rearrangement, and enlargement o the cells, Savage (1956) concluded that the sequence noted in *Pieris* (Henso 1937), in which the phase of cell proliferation precedes the phase of cell rearrangement and enlargement, is more advanced than that of *Blatta* (Henso 1944) and *Schistocerca* (Savage, 1956) in which cell proliferation and rearrangement occur before cell enlargement. This view appears to be based on th ground that the former type of sequence is seen in more advanced insects In *Dysdercus* 3 phases are noted—those of cell production, of cell productio and rearrangement, and of cell rearrangement and enlargement. Thus the processes are overlapping and somewhat similar to those of *Pieris*. But *Forficul* (Henson, 1946*a*) also resembles *Pieris* in this respect, and so this type o sequence cannot be considered advanced. The question really seems to b related to the presence or absence of secondary Malpighian tubules: in insect in which secondaries are absent (e.g. *Pieris*, *Dysdercus*), the Malpighian tubule ontinue to elongate for a long time and cell rearrangement and enlarge- ent continue together, whereas in insects in which secondaries make their ppearance to meet the increasing excretory requirement, cell enlargement ccurs only for a short time after cell rearrangement has been completed.

Wheeler (1893) regarded the primitive number of Malpighian tubules in nsects to be 6, but Henson (1944) believed that the primitive condition con- ists of 6 primary and numerous secondary tubules, the development of the atter being suppressed in the higher Pterygota. Savage (1956) thought that *chistocerca* shows the latter condition. It is true that the secondaries are seen n lower insects, but when present, they develop much later than the pri- aries. Besides, *Schistocerca* possesses a larger number of secondaries than *latta* and *Forficula*. Therefore it seems possible that the primary and secon- ary tubules do not have an identical nature, and that the primaries, appearing uch earlier in ontogeny, represent the primitive tubules. If so, Wheeler's oncept regarding the primitive number of tubules would be correct, and the ddition of the secondaries may be related to the increased excretory require- ent which cannot be met by the primaries.

In *Blatta*, *Pieris*, and *Schistocerca* the striated border develops on the inner argin of the cells during embryonic development, while in *Dysdercus* this evelopment and the differentiation of the proximal and distal regions takes lace in the first nymphal stage. Evidently the true embryonic life does not rminate with emergence, but continues in the first nymph. Such a view was xpressed earlier by Henson (1929, 1946). Further changes involving growth nd differentiation are cyclical and repeated in each nymphal stage, as ob- rved already in *Blatta*, *Forficula* (Henson, 1944, 1946), and *Schistocerca* Savage, 1956). But while the increase in the size of the nucleus is gradual in *ysdercus* and *Schistocerca*, it occurs suddenly in the third instar in *Forficula* nd in *Pieris* it is accompanied with lobulation and ramification. These ifferences are not properly understood, but it is not unlikely that the increase the nuclear size or surface area is related to the increase in the size of the lls.

A few words may be added here about the appearance of granules in the istal region of the tubules in each nymphal instar as it starts feeding. These e flushed into the lumen towards the end of each instar, pass into the hind- ut and contribute to the formation of the meconium. The appearance of the anules in the active, feeding nymph and their elimination at each moult ow that they are excretory products.

APPENDIX

TABLE 1

Condition of the embryonic Malpighian tubules at various stages of development of the embryo

Age of embryo	*Length of tubules* (mm)	*Diameter of tubules* (μ)	*No. of cells in a T.S.*	*Size of nucleus in each cell* (μ)	*Appearance of vesicles*	*Whether tubules free or fused*
9 days, 3 h	0·04	36	17–20	5·4×3·6	vesicle absent	free
9 days, 8 h	0·09	36	up to 20	"	"	joined at tips but lumen separate
9 days, 12 h	0·108	28	12–15	4·8×4·8	formation starts	"
9 days, 16 h	0·136	25	10–12	4·8×4·8	vesicle formed	"
10 days, 10 h	0·256	19 to 22	8–10	4×4	vesicle present	"
10 days, 16 h	0·288	"	"	"	"	"
11 days	0·352	"	"	"	"	"
11 days, 12 h	0·800	14	6–8	"	"	"
12 days*	1·92	"	"	"	"	fusion complete; lumen continuous
12 days, 22 h	2·88	13	6	3·2×3·2	"	"
13 days, 12 h	3·20	"	3–4	"	"	"
14 days, 3 h	3·52	"	3	3·2×2·4	"	"
14 days, 17 h (a few h before hatching)	"	"	"	"	"	"

* At this stage the two tubules of a pair have united to form a loop. Therefore subsequent figures relate to the measurement of a loop.

TABLE 2

Gradual increase in the length of the Malpighian tubules in various nymphal stages

Nymphal stage	*Length of a loop of Malpighian tubules in the young nymph* (mm)	*Length of a loop in the middle-aged nymph* (mm)	*Length of a loop in the old nymph* (mm)
1st nymph	3·5	4·2	4·5
2nd nymph	4·5	6·4	7·4
3rd nymph	8·1	10·6	11·2
4th nymph	12·8	14·7	15·0
5th nymph	15·2	22·7	30·2

TABLE 3*

Diameter of the Malpighian tubules at different stages of life-history

1st nymph	μ	*4th nymph*	μ
Newly hatched	16	After last ecdysis	58
12 h after hatching	22	After 12 h	79
2 days and 12 h old	25	3 days later	83
Immediately before ecdysis	22	1 day before ecdysis	79
2nd nymph		Immediately before ecdysis	72
Soon after last ecdysis	22	*5th nymph*	
2 days later	29	After last ecdysis	72
4 days later	40	1 day later	83
6 days later	29	2 days later	94
Immediately before ecdysis	29	Old nymph	101
3rd nymph			
Soon after last ecdysis	40		
3 days later	54		
After 6 days, 12 h	61		
Immediately before ecdysis	58		

* All measurements have been taken from whole mounts and in the distal region.

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Structural Changes in the Gills, Intestine, and Kidney of *Etroplus maculatus* (Teleostei) adapted to different Salinities

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SUMMARY

There is an increase in the number of the so-called 'chloride cells' in the gill epithelium with increasing salinity of the medium. In 75% and 100% sea-water the 'chloride cells' exhibit a change in structure, in that they become more granular and acquire an apical vacuole. There is also a general decrease in the number of mucous cells with increasing salinity of the medium of adaptation.

The glomerulus tends to become smaller in the fishes adapted to high salinity as compared to the fresh-water fish. There is also a deposition of pigment around the nucleus and an indication of some changes in the nucleus of the kidney tubule cells in fishes adapted to 100% sea-water.

In the intestine, adaptation to high salinities results in an increase in the nuclear size of the columnar epithelial cells, an increase in the number of flask-shaped cells, and a considerable increase in the thickness occupied by the tunica propria.

INTRODUCTION

IT has been shown by Pampapathi Rao (1958) that the fresh-water teleost, *Etroplus maculatus*, can be adapted to 100% sea-water by gradual transfer from fresh water through intermediate salinities. It is well known that the fresh-water fishes keep up the hypertonicity of their blood mainly by the help of the water-excreting kidney. But some loss of salts through urine cannot be avoided, and this is usually made good either through food or through the active absorption of chlorides through the gills against a gradient, and also to a certain extent through resorption of salts in the distal segment of the kidney. But the whole condition is reversed in the case of marine teleosts, where the water-excreting kidney becomes a liability, since the fish has got to maintain its internal environment hypotonic to the medium. In marine teleosts water lost through osmotic conditions is compensated by swallowing sea-water. The sea-water contains considerable amount of salts which the fish has to get rid of, and Smith (1930) showed that the ion balance is kept up by the continuous extrarenal excretion of salt in addition to renal excretion. Keys and Willmer (1932) first discovered the large acidophil cells on the gill-tissue of the eel, which they named 'chloride cells'. They suggested that these cells have an important role to play in salt excretion. Similar cells were observed by Copeland (1948) in the gills of *Fundulus*, and by Getman (1950) in the gill epithelium of *Anguilla*. Morris (1957) described similar cells in *Lampetra fluviatilis*, before entry into fresh water.

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In fish, therefore, osmotic regulation involves water-excretion by the kidney and salt-uptake by the gills in hypotonic media, while in hypertonic media there is water-retention by the kidney, salt-excretion by the kidney as well as the gills, and water-uptake through the agency of the intestine.

While there are some studies on the structural changes in the gill epithelium with change in the osmotic concentration of the medium (see Morris, 1957, for a review), there are few studies along similar lines with regard to the changes in the kidney and scarcely any studies on the changes in the intestine. The structure of the kidney of fishes has been extensively studied in relation to the natural habitat of the organism (Smith, 1930, 1932; Grafflin, 1937; Tampi, 1959).

It will, therefore, be of interest to know the structural changes that these three principal organs (namely the gill epithelium, the kidney, and the intestinal epithelium) undergo in a fish such as *E. maculatus*, which normally lives in fresh water (hypotonic medium), when it is adapted to sea-water (hypertonic medium).

The present paper records the results of such a study.

MATERIALS AND METHODS

E. maculatus was collected from local fresh-water ponds, and stocked in a large tank in the laboratory. To begin with, 16 fish were put in 30% sea-water. After 4 days 12 of them were transferred to 50% sea-water, and the remaining 4 left in 30% sea-water for 3 weeks. Again after 4 days 8 of the fish from 50% sea-water were transferred to 75% sea-water, and the remaining 4 were adapted to 50% sea-water for 3 weeks; of the 8 fish put in 75% sea-water, 4 were transferred to 100% sea-water after 4 days, and the remaining 4 adapted to 75% sea-water for 3 weeks. The 4 fish transferred to 100% sea-water were left in that medium for 3 weeks, as in the other media.

All the fish which were kept in different media were fed every day with rice and bits of earthworm.

Simultaneously, some fish were maintained as controls in the fresh-water medium. The gills, kidneys, and intestine of these were fixed in Bouin's fluid, dehydrated in alcohol, embedded in paraffin, and sectioned at 6 μ, and stained with Mallory's triple stain or iron haematoxylin and eosin. Similarly, gills, kidneys, and intestine of fish adapted to various salinities were fixed in Bouin's fluid made up in sea-water, sectioned, and stained separately. The glomerular diameter was measured in freshly mounted tubules from macerated kidneys of fish adapted to fresh water and to 100% sea-water. Several such tubules were measured and the average taken.

RESULTS

Gill epithelium. Each gill filament is supported by a skeletal axis. The gill epithelium is primarily made up of basal and platelet cells, mucous cells, and 'chloride cells' (fig. 1, A). Mucous cells are scattered throughout the normal gill epithelium of fresh-water fishes, mainly towards the tip and base of the

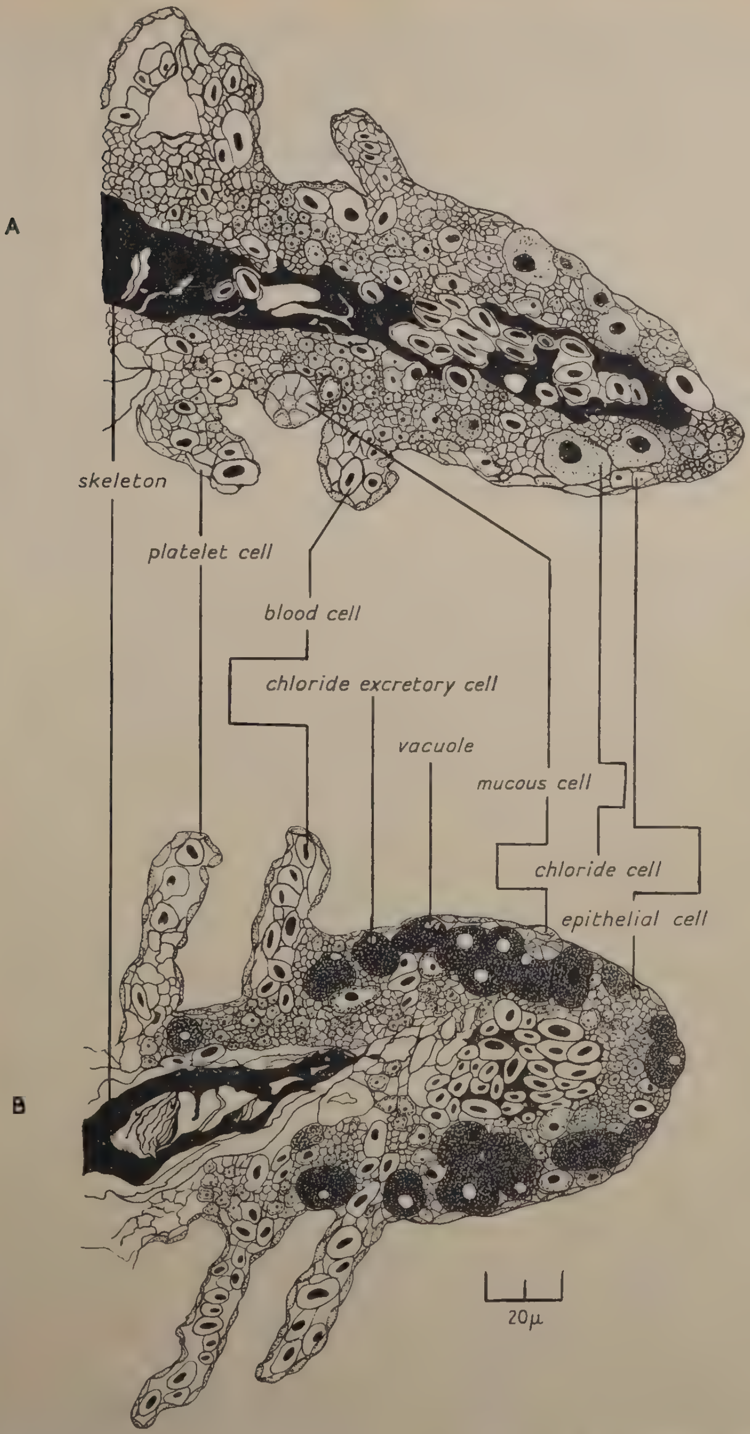

FIG. 1. Sections showing the gill epithelia of *E. maculatus* from fresh water (A) and from 100% sea-water (B).

filaments. They stain light blue in Mallory's triple stain and appear vesicular. In addition to these cells there are other cells with the nucleus stained red, and the cytoplasm blue and granulated in appearance. Some of these cells are oval and some are round or irregular in shape. The nucleus of the cell is situated towards the centre of the cell. They resemble similar cells described by earlier workers in various fresh-water teleosts and also recently in the lampern by Morris (1957). It is thought that these cells are responsible for the active uptake of salt from the hypotonic medium and have been named 'chloride cells'. Whether they have this function is not known, but the name will be used in this paper. These cells are present over most parts of the gill epithelium, usually in the interplatelet areas.

With adaptation to increased salinity (i.e. 30% sea-water) interesting changes in the structure of the gill epithelium are noticed. An increase in the size (table 1) and number of the chloride cells has been observed. Interestingly enough the size of the mucous cells is also seen to be increasing. No change has been observed in the cytoplasmic granulations of the chloride cells.

In the sections of the gills of the fish adapted to 50% sea-water it has been noted that the number and size of the chloride cells increased further. Against this the mucous cells showed a decrease in their numbers.

In 75% sea-water it has been noted that the number and size of the chloride cells is the same as in 50% sea-water. However, towards their periphery a vacuole was seen and their granulation was found to be increasing. It has been observed that the chloride cells present in the interfilamentar junctions were slightly enlarged. The mucous cells showed a steady increase in their size and a decrease in numbers.

In the fish adapted to 100% sea-water there was a decrease in size of the chloride cells, the diameter varying between 3·6 μ to 5·4 μ. But the chloride cells present in the interfilamentar junctions were found to have increased in size. The granulation of these cells was much increased. A vacuole was found towards their periphery (fig. 1, B). The mucous cells had been reduced to negligible numbers.

Intestine. The following layers are seen in transverse sections of the intestine of *E. maculatus* adapted to fresh water (fig. 2, A). The outermost layer of the intestine is the serosa. Then follows the muscular layer consisting of the circular and longitudinal muscle-fibres. Just beneath this is the stratum granulosum. It is followed by a thin tunica propria, stained deep blue; this extends into the spaces of the rugae. Then comes the last layer, the epithelial layer, with characteristic columnar cells with their nuclei stained red with Mallory. The luminal ends of the columnar epithelial cells of the intestine are densely stained. There are a few goblet cells in the columnar epithelial layer, opening into the lumen of the intestine. They stain light blue. Most probably these are mucous cells.

One interesting feature observed in the structure of the intestine is that with the increase in salinity from 30% to 100% sea-water the tunica propria

TABLE 1

Changes in the size of cells in the gill epithelium and kidney of E. maculatus *on adaptation to media of increasing salinity.*
(All figures are in μ.)

Medium to which adapted	*Gill Epithelium*				*Kidney*							
	Diam. of chloride cell		*Diam. of mucous cell*		*Outer diam. of tubule*		*Diam. of lumen*		*Diam. of tubule cell*		*Maximal diam. of glomerulus*	
	Mean	*S.D. ±*	*Mean*	*S.D. ±*	*Mean*	*S.D. ±*	*Mean*	*S.D. ±*	*Mean*	*S.D. ±*	*Mean*	*S.D. ±*
Fresh water . . .	2·9	1·3	5·6	1·3	17·6	4·1	5·2	1·5	13·5	1·2	90·0	22·4
30% sea-water . .	4·4	1·5	7·5	1·8	10·4	4·2	3·6	1·8	9·9	7·3	—	—
50% sea-water . .	6·6	1·2	7·9	1·4	16·6	4·3	4·4	1·9	12·2	3·2	—	—
75% sea-water . .	6·4	1·0	8·4	1·7	16·9	6·0	4·0	1·8	13·0	3·7	—	—
100% sea-water . .	4·5	0·9	—	—	14·0	5·7	4·9	2·3	9·2	2·6	71·4	20·3

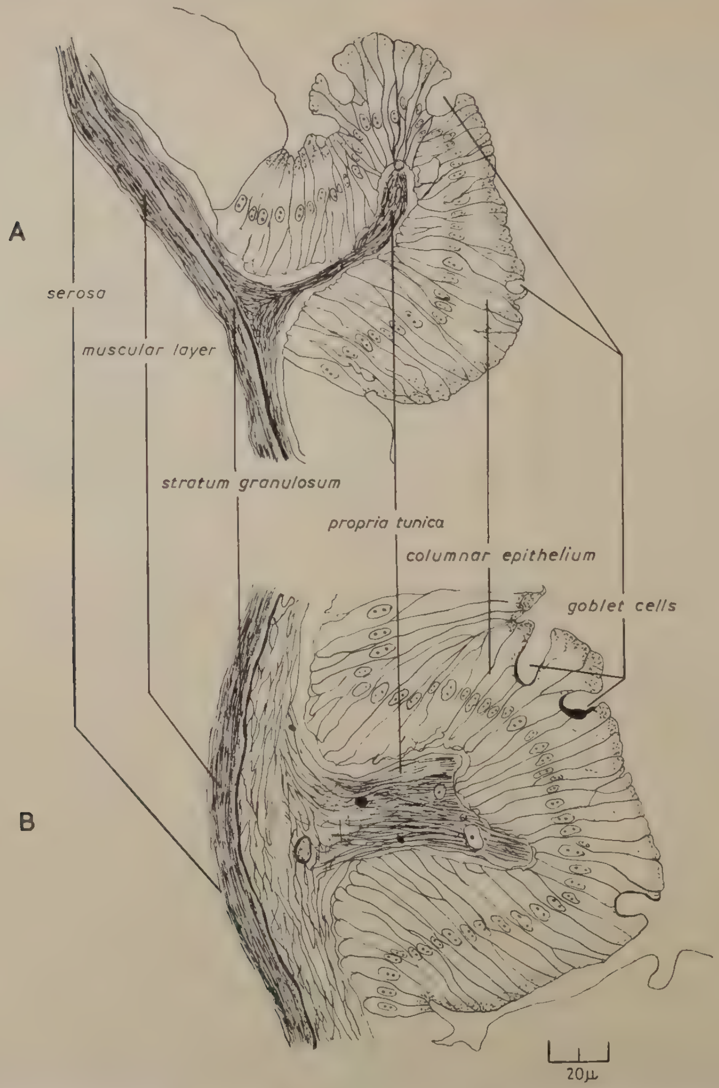

Fig. 2. Transverse section of the intestine of *E. maculatus* adapted to fresh water (A) and to 50% sea-water (B).

becomes much wider (figs. 2, 3). Further, it was also found that with increasing salinity there was an increase in nuclear size, and an increase in numbers of goblet cells present in the columnar epithelial layer.

Kidney. The following structure was noticed in the sections of the kidney of fish kept in fresh water. The kidney cells have a uniformly stained cytoplasm with nuclei towards the centre. The lumen is intercellular and the diameter of the lumen ranges from 3·6 μ to 7·2 μ. The external diameter of the tubule varies from 12·6 μ to 23·4 μ, and the size of the tubule cells ranges from 9·9 μ to 14·4 μ. The kidney cells are narrow towards the lumen and broad towards their outer side. The glomerular diameter of the fish adapted to fresh water is about 90 μ.

Several changes are noticed in the structure of the kidney as a result of adaptation to changes in salinity. In fish adapted to 30% sea-water the lumen is very much reduced in many tubules, but quite open in some. The cytoplasm present towards the lumen of the cell is densely stained and that towards the periphery is clear. The nucleus is at the centre of the cell. A decrease in the diameter of the lumen is observed. The diameter ranges from 1·8 μ to 5·4 μ. The external diameter as well as the cell size are reduced (table 1).

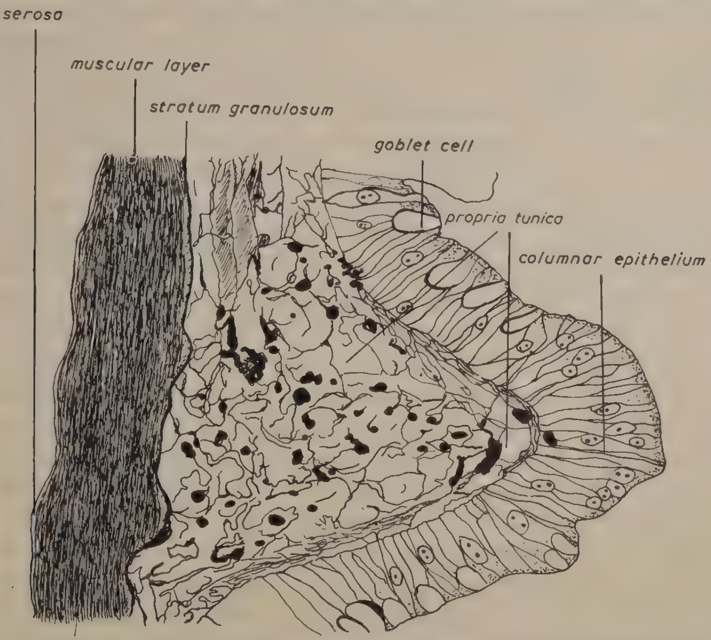

FIG. 3. Transverse section of the intestine of *E. maculatus* adapted to 100% sea-water.

In sections of the kidney of fish adapted to 50% sea-water a large granule of black pigment was found to have accumulated inside almost all the cells, and the nucleus could not be distinguished in these cells. In some cells numerous black granules were found, probably in the process of formation of the big black pigment granule inside the cell. The outer diameter of the tubule, the lumen diameter, and the cell size are like those of fishes in fresh water (table 1).

In 75% sea-water the accumulation of black pigment inside the cells was increased. There are no significant differences in other respects from what is seen in the kidneys of fishes adapted to 50% sea-water.

In 100% sea-water a vacuole was noticed inside the cell in some cells, and wherever there was a vacuole the nucleus was absent. The formation of the vacuole is a very peculiar phenomenon. There was a decrease in the cell size. The glomerular diameter showed a decrease to about 71·4 μ. Sometimes the tubule did not appear to end in a glomerulus.

DISCUSSION

The results presented above show that adaptation of *E. maculatus* to increasing grades of sea-water results in changes in two main features of the gill epithelium. One important change concerns the size and structure of the granulated pink-staining cells. When the fish is adapted to 30% sea-water there is a noticeable increase in the size of the granulated cells, and they also appear to be more abundant. In this medium there would not be a need for active uptake of salt from the medium. It is not clear what function they perform. It might be that they are the precursors of the chloride excretory cells, and are derived from the chloride cells of the fresh-water fish, since they are not fundamentally different in structure, although they are larger and a little more densely granulated. The same trends are increasingly seen in fish adapted to 50% sea-water. It is possible that in this medium they are already concerned with excretion of salt. In fish adapted to 75% and 100% sea-water the chloride cells exhibit structural changes, the most conspicuous being the appearance of a vacuole at the apical end of the cell. The granulation is denser. The vacuolated cell does not appear to be a new cell-type, but rather a derivative of the granulated chloride cell. Such cells bearing apical vacuoles have already been described in marine *Fundulus heteroclitus* (Copeland, 1948). It has been suggested by Copeland that these cells are concerned with salt excretion and that they are derived from the chloride cells of fish adapted to fresh water. There is no evidence against such a transformation and the present results support Copeland's view. Instead o secreting inwards, the cells would now secrete outwards, thus reversing thei physiological polarity. However, Parry, Holliday, and Blaxter (1959) have recently obtained some evidence which indicates that those acidophil cells may not be very important in salt regulation.

There is a considerable increase in the size of the individual mucous cel of the gill epithelium with increase in the salinity of the medium. But th number of mucous cells decreases conspicuously with increase in salinity. I might be that mucous requirements in fresh water are more important (i reducing inward permeability to water) than in sea-water.

It is likely that when the fresh-water *E. maculatus* is transferred to sea-water the intestine would take up the osmotic functions that are well know in marine teleosts. The results reported above indicate that there is some chang in the structure of the intestine on adaptation to hypertonic media. The mai changes are noticed in the columnar epithelium and the tunica propria, bot of which increase in thickness. The nuclear size of the columnar cells als increases. Whether all these changes reflect any increase in the water-absorp tive function of the intestine is not established. The significance of th increase in the number of goblet cells is also not clear.

The changes in the kidney are noteworthy. The most conspicuous change is the reduction in glomerular size with adaptation to sea-water. It might be that this reduction in glomerular size is a direct result of the decreased need for filtration. Such a view would be in agreement with Smith (1932). However, it has been shown that filtration does not depend much on the osmotic gradient, but that the hydrostatic pressure in the capillaries and the total filtering surface are the important factors influencing filtration. In the present study no estimate of the total filtering surface is made. Hence it could not be said at present whether the decrease in glomerular size in fish adapted to sea-water results in a reduction in the total filtering area. But under the conditions in which the glomerular size changed, it is logical to assume that the total filtering surface is decreased. The changes in the sizes of cells and lumen are also to be looked upon as consequences of changes in kidney function. That the physiology of the tubule cells is affected may be noticed from the nuclear changes described above.

I am grateful to Professor Kandula Pampapathi Rao, Head of the Department of Zoology, for suggesting the problem and for his help and guidance throughout the course of this investigation. My thanks are due to Mr. P. Sitaramiah of this Department for assistance in statistical analysis of the data, and to Mr. V. Chandrasekar for his assistance in drawing.

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The Histochemistry of the Radula of *Patella vulgata*

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SUMMARY

When first secreted the radula consists of chitin and protein. In the bases and cusps of the teeth the reactions for tyrosine, tryptophane, and chitin become negative, and are replaced by reactions for amino groups and argentaffin material. The bases of the teeth become impregnated with iron. Subsequently the reactions for amino groups and argentaffin material become negative in the cusps and they then become impregnated with iron and silica. Very few changes have been observed in the radular membrane.

It is suggested that the occurrence of quinone tanning might account for these histochemical changes observed in the radula, but phenols derived from tryptophane would appear to be more important in the tanning of the radula than those derived from tyrosine.

INTRODUCTION

IN view of the great importance of the radula in the life of the gastropod and cephalopod molluscs it is surprising that the composition of this organ has been so neglected. A vast amount of work on its anatomy has been carried out owing to its importance in systematics. The growth and mode of secretion of he radula has been investigated by many authors, but the results have so far been very inconclusive. Thanks largely to the work of Spek (1921), Sollas 1907), Jones and others (1935), and Rudall (1955) there is little doubt that the radula contains chitin, protein tyrosine, and various minerals. In *Patella vulgata* large amounts of iron (14·3% dry wt. Fe_2O_3) and silica (8·7% dry wt. SiO_2) are found. Gabe and Prenant have carried out very extensive investigations of the molluscan radula but mainly on the cytology. Recently (1952) they have published the results of some histochemical studies and they conclude that mucopolysaccharides are present. It was decided therefore to investigate histochemically, first the radula of *P. vulgata* (the results of which form the substance of the present paper) and then to extend this study to various other molluscan species (the results to be published as a subsequent paper).

MATERIAL AND METHODS

Limpets (*P. vulgata* L.) were obtained from the Scottish Marine Biological Association, Millport. The radula of an adult limpet is between 5 and 8 cm long, and it is impossible to section most of this owing to its extreme hardness. Two pieces, each 1 cm in length, were cut therefore from the proximal end, nd fixed. The majority of this investigation was carried out on material fixed in formaldehyde-calcium (Baker, 1944); where extraction of lipids was necessary weak Bouin's fixative was used (Baker, 1946), and for microincineration lcoholic buffered formalin (Glick, 1949). This material was then embedded in ster wax (Steedman, 1947) and sectioned at 8 μ. For the investigation of lipids

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material fixed in formaldehyde-calcium was embedded in gelatin and sectioned on a freezing microtome (Baker, 1944).

Microincineration was carried out by the procedure given by Glick (1949). After incineration the slides were covered with a coverglass, ringed with paraffin wax, and examined by dark ground illumination. Some attempt was made to study the distribution of silica by the methods given by Sollas (1907).

The presence of chitin was investigated by microchemical techniques (Gatenby and others, 1953), and by paper chromatography (Smith, 1958).

The histochemical methods employed in this study are summarized in table 1, and the various controls and blocking reactions in table 2 (see appendix).

Attempts were made to investigate the stabilization of the protein in the radula by the methods suggested by Brown (1950*a*).

For the investigation of the effects of diaphanol on Mallory staining of the radula the material was fixed in formaldehyde-calcium, washed in water and 75% alcohol, and then placed in the diaphanol. The control and the pieces subjected to the action of diaphanol for 1, 2, 3, 4, 6, 8, or 11 days were washed in 75% alcohol and then embedded in ester wax.

RESULTS

The results of the histochemical reactions are summarized in fig. 1.

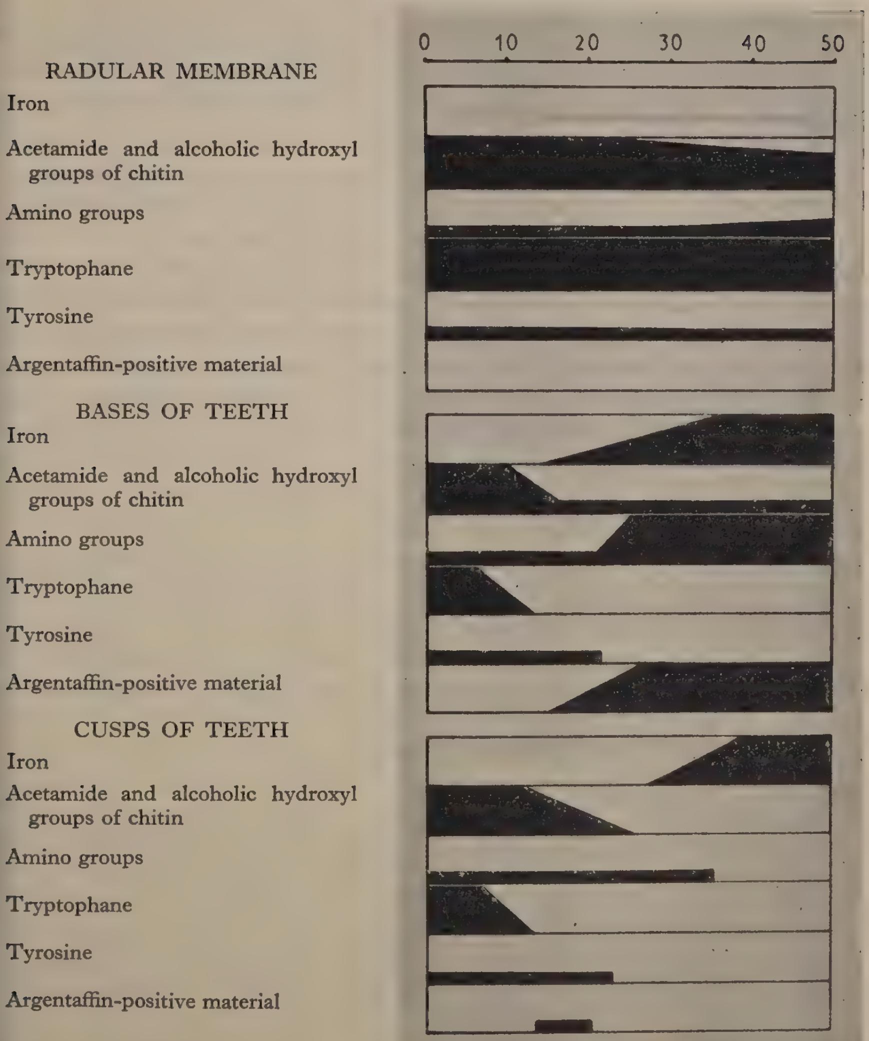
Minerals

By using Sollas's procedure for investigating silica it was found that after the nitric acid treatment only cusps and small pieces of the bases of the teeth remained as a brown sludge. Treatment of the sludge with aqua regia dissolved the remains of the bases and also released iron from the cusps, as was shown by the reaction of the filtrate with potassium thiocyanate solution. The filtrate did not react with ammonium molybdate solution (Vogel, 1954), so that soluble silicates were absent. The residue consisted of colourless material which dissolved in hydrofluoric acid and was presumably the hydrated silica shown to be present by Sollas (1907). It was of interest, however, that the residue consisted of the posterior surfaces only of the older cusps. The methods employed did not allow any determination of the level along the radula at which silica appeared to be made.

Microincineration revealed a dense red ash in the cusps from row 45 onwards. Iron is the only mineral giving a red ash with this procedure (Glick, 1949). With the use of the Perl technique iron was shown to appear in the teeth bases from row 13 onwards and the teeth cusps from row 28. It never appeared in the radular membrane, however.

Carbohydrates

The microchemical reactions confirmed the presence of chitin in the radula, and chromatography has shown that the only sugar present in 0·1 N sulphuric acid hydrolysates of the radula is glucosamine. Chitin therefore



IG. 1. A summary of the histochemistry of the radula of *P. vulgata*. The numbers refer to he tooth row, numbering from the proximal end of the radula. The thickness of the black in is proportional to the intensity of the reaction.

ppears to be the only carbohydrate present in the limpet radula. The histo-hemical results for carbohydrates are in agreement with this conclusion. hese results are analysed in detail elsewhere (Runham, 1960*b*) and only a ummary is given here. The acetylamide-glycol groups of chitin are capable of iving a positive PAS reaction at least in this material. The alcian blue and ale reactions are both due to intermolecular chelation between the acetyl roup and the alcoholic hydroxyl group. Thus acetylation by blocking the ydroxyl group, and methylation which has been shown to hydrolyse the acetyl group, prevent these reactions. Methylation releases amino groups, as shown by the bromophenol blue reaction. Deamination particularly after methylation causes hydrolysis of the chitin and probably conversion of the glucosamine to anhydro 2:5 D mannose, thus decreasing the intensity of the PAS reaction.

Proteins

The bromophenol blue reaction by means of a deamination control has been found to be specific for amino groups. The hydroxynaphthaldehyde reaction reveals only protein α acyl amino groups, and the amino groups revealed by this reaction largely parallel those obtained with the bromophenol blue technique. The ninhydrin / Schiff reaction was found to be very weak, so that only the presence of large numbers of amino groups was revealed.

The Morel–Sisley reaction paralleled the results obtained with the Millon reaction, but was far more intense.

Lipids

The reactions for lipids in the radula were all negative. It is possible that bound lipids are present although this is unlikely in view of the recent criticisms of Berenbaum's technique by Locke (1959). Locke has shown that chitosan will give the sudan black B / acetone reaction for bound lipids, and it is of interest that in this material the regions containing free chitin appeared to be stained by this technique.

Other compounds

With the diazonium reaction a fiery red coloration indicates the presence of polyphenolic material. In the case of the radula only a yellow to orange colour, rather more intense in the bases of the older teeth, was found: it was assumed that this reaction was negative. The argentaffin reaction was strongly positive in the older bases. A detailed investigation into the basis of the argentaffin reaction in this material is necessary before any definite conclusions can be drawn.

It was decided to investigate the possibility that the secretion of iron into the radula was responsible for some of the changes in the histochemical reactions. The results were inconclusive. The sections were treated for 24 h at room temperature with the solution devised by Warner and Weber (1953) for their studies on the iron binding of proteins. Subsequent to this treatment the Perl reaction revealed that the iron was distributed in a similar fashion to that found in the Hale reaction. The iron treatment blocked the alcian blue reaction, which indicated that the same chemical groups are responsible for the Hale and the alcian blue reaction in this material. The iron solution caused an increase in the bromophenol blue staining, an effect which warrants more detailed investigation. It is possible that iron secreted into the radula might cause the increase in the number of amino groups in the bases of the older teeth, but as a similar sequence occurs in other moluscan radulae where iron is not found, this is considered to be unlikely (Runham, 1960*a*).

By use of the series of solutions suggested by Brown (1950*a*) for the investigation of the forces stabilizing proteins it was found that the radula was affected only by the sodium hypochlorite solution. This would suggest the presence of quinone-tanned material.

Histochemical reactions for tyrosinase were negative.

Discussion

The radula when first secreted consists of chitin and protein, possibly in the form of a glycoprotein, as in the insect cuticle (Hackman, 1959). The acetamide and hydroxyl groups of the chitin are at least in part available for reaction, while the protein gives weak reactions for amino groups and tyrosine, and very strong reactions for tryptophane. Subsequently the radula becomes altered chemically in various ways. The end-result of these changes is a radula consisting of exceptionally hard radular teeth mounted on hard but brittle bases, and these are embedded in a tough, leathery, radular membrane.

In the invertebrates quinone tanning is a common method of hardening (Brown, 1950*b*). It was therefore of great interest to find out to what extent the histochemical results obtained here indicate that quinone tanning occurs in the radula.

Previously described examples of quinone tanning have the following properties in common: the enzyme responsible both for hydroxylation and for quinone formation is the phenolase complex (Mason, 1955); phenolic or polyphenolic material is present (perhaps in some cases aminophenols (Pryor, 1955); on exposure to air the untanned material often darkens; and the tanned material is soluble only in the sodium hypochlorite solution of Brown's series of solutions.

All attempts to demonstrate phenolase histochemically in the radula have given negative results. Further investigations must be carried out before this onclusion can be accepted. From the work of Whitehead and others (1960) nd of Yasonobu (1959) it would appear that phenolases from different sources have differing substrate specificities. Therefore methods involving the use of atechol (Smyth, 1954) and haematoxylin (Lillie, 1956) as substrates may not emonstrate the enzyme in the molluscan radula. Further work has to be carried out with a wider range of substrates.

The argentaffin reaction was intensely positive in the bases of the older eeth, while the diazonium reaction was negative. Both of these reactions eveal aminophenols and polyphenols (Lison, 1936). In the absence of investiations on the specificity of the reactions, the meaning of these results is somewhat uncertain. Smyth and Clegg (1959) found that in cestodes one diazonium alt gave a strong reaction with the polyphenols of some species but not with thers. Only fast red salt B has been used in this study. If a soluble phenol were involved it would presumably have dissolved out in the preparatory proedures. If aromatic amino-acids in the protein of the radula were oxidized by a phenolase to quinones they might react with the available amino and/or sulphydryl groups of other protein chains as soon as they were formed, so that it may not be possible to detect them histochemically.

Exposure to air does not appear to cause a darkening of the limpet radula, but, as the only darkening in the radula of this species appears to be due to iron, this is to be expected. Of the series of reagents suggested by Brown for the identification of the forces stabilizing proteins, only sodium hypochlorite solution dissolved the radula. Boiling in saturated potassium hydroxide for 1 h does not affect the shape of the radula but dissolves out most of the protein, leaving the chitin. That only the sodium hypochlorite dissolved the radula implies that covalent bonds are responsible for its stabilization, but in this case they may well be the covalent bonds of chitin, not of a quinone-tanned protein. Experiments were carried out on the cuticle of *Locusta* to confirm these results. After exposure to the 10% sodium hypochlorite solution for 1 day the cuticle was bleached and after 2 days it had disintegrated into small pieces of which very few remained after 3 days.

On these criteria the evidence for the presence of quinone tanning in the radula is very slight and equivocal. There is, however, some indirect evidence that quinone tanning may occur here. Dennell and Malek (1955 *a*, *b*) divide the tanning of the insect cuticle into primary and secondary tanning thus:

Primary tanning

1. Sterol reactions at first positive become negative.
2. Protein tyrosine at first positive becomes negative.
3. Protein amino groups at first strongly positive become weaker.
4. An argentaffin reaction appears.
5. Mallory's triple stain stains the cuticle initially blue but this is later replaced by red staining. This change is reversed by treatment with diaphanol which destroys the aromatic amino-acids.

Secondary tanning

1. The red Mallory staining disappears, leaving the cuticle unstained.
2. The weak reactions for protein amino groups become negative.

Apart from the presence of sterols all these changes are found in the radula of the limpet. Diaphanol caused a gradual reversal of the changes in Mallory staining, the reversal being complete after 6 days' treatment. Kennaugh (1957) found that diaphanol first removed the aromatic acids from the insect cuticle and then more slowly the other ninhydrin-positive material. It would therefore appear that aromatic groups are responsible for the change in Mallory staining of the older radula. As the resemblances between the insect cuticle undergoing tanning and the radula are so great, it would seem likely that a similar mechanism is involved. In the radula, however, it would appear that quantitatively tryptophane is more important than tyrosine in hardening.

The sequence of changes that the author envisages as occurring in the radula of *P. vulgata* is as follows. The radula is first secreted as a chitin-protein mixture or as a glycoprotein (Hackman, 1959). As far as has been observed in this species very few subsequent changes occur in the radular membrane. The bases of the teeth become impregnated with extra protein material rich in amino groups and this becomes linked to the structural protein by its tyrosine and trytophane groups, presumably through phenolic derivatives. The cusps appear to be similar to the bases of the teeth, but either tanning is more rapidly completed, or the tanned material becomes linked to the impregnating silica. It is not known which of these two alternatives is the more likely.

Previously described evidences of quinone tanning are believed to involve tyrosine derivatives and are therefore related to melanin formation. However, there is a group of pigments based on oxidation products of tryptophane, namely the ommins (Forrest, 1959). A system of tanning based on tryptophane derivatives would appear to be possible and has previously been suggested by Pryor (1955). The oxidation product of tryptophane found in the ommins is 3-hydroxy kyneurenine. If this oxidation occurred in the radula one could explain the disappearance of reactions for tryptophane with the simultaneous appearance of reactions for amino groups and the argentaffin reaction. It is proposed to carry out a chemical investigation to determine if in fact this mechanism of tanning occurs in the radula.

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APPENDIX

TABLE 1. *Summary of histochemical reactions*

Reaction	*Reference*	*Material demonstrated*
Peri	Bunting, 1949	ferric iron
periodic acid / Schiff (PAS)	Pearse, 1953	glycol or glycolamine groups of polysaccharides
alcian blue	Zugibe and others, 1959	acid groups of polysaccharides
Hale	Pearse, 1953	acid groups of polysaccharides
mucihaematin	Casselman, 1959	mucin
azure A metachromasia	Kramer and Windrum, 1955	acid groups of polysaccharides
sulphation, azure A	Kramer and Windrum, 1954	free hydroxyl groups
bromophenol blue	Mazia and others, 1953	amino groups
ninhydrin / Schiff	Burstone, 1955	amino groups
hydroxynaphthaldehyde	Weiss and others, 1954	protein amino groups
rosindole	Glenner, 1957	tryptophane
Morel / Sisley	Lillie, 1957	tyrosine
Millon	Pearse, 1953	tyrosine
Sudan black B / 70% alc. (SBB)	Pearse, 1953	lipids
Nile blue	Pearse, 1953	acid lipids
burnt SBB	Berenbaum, 1958	bound lipids
SBB / 70% alc. 70° C	Berenbaum, 1958	bound lipids
SBB / acetone 70° C	Berenbaum, 1958	bound lipids
diazonium	Pearse, 1953	polyphenols
argentaffin	Lison, 1936	polyphenols, polyamines, &c.
aldehyde	Danielli, 1950	aldehydes
tyrosinase	Smyth, 1954	tyrosinase
	Lillie, 1956	tyrosinase

TABLE 2. *Summary of control procedures*

Control reaction	*Reference*	*Material affected*	*Comments*
diastase		glycogen	1% BDH diastase in aq. dest. 37° C ½ h
hyaluronidase	Pearse, 1953	hyaluronic acid; chondroitin sulphuric acids A and C	Benger's testicular hyaluronidase
pectinase	Grainger and Shillitoe, 1951	galactogen and pectin	obtained from N. W. Carter
acetylation	Lillie, 1954	amino and hydroxyl groups	
benzoylation	Lillie, 1954	amino and hydroxyl groups	
deacetylation	Pearse, 1953	acetyl groups	
deamination	Lillie, 1954	amino groups	
methylation	Fullmer and Lillie, 1957	acid groups	
demethylation	Spicer and Lillie, 1959	methyl esters	
aldehyde blockade		aldehyde groups	
pyridine extraction	Baker, 1946	lipids	material fixed in weak Bouin
iron solution	Warner and Weber, 1953		

Lipids in the Human Oocyte

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SUMMARY

The cytoplasm of the human oocyte contains two categories of lipid bodies that are here called L_1 and L_2 for the sake of brevity. The L_1 granules and spheres consist of phospholipids and triglycerides. The L_2 bodies occur as aggregations of granules or fenestrated plates, consisting of phospholipids. The yolk granules are made up of phospholipids, proteins, and carbohydrates.

INTRODUCTION

BY applying histochemical and 'Golgi' techniques the present author has studied various lipid-containing bodies in the oocytes of several reptiles, birds, and mammals (Guraya, 1957, 1958, 1959*a*, *b*, *c*, *d*, *e*, 1960, 1961). He has already summarized the previous work on the morphology of cell components in the mammalian egg (Guraya, 1959*a*). Beams and Sheehan (1941) have described the Golgi material in the form of irregular bodies and mitochondria in the form of granules in the human oocyte.

MATERIAL AND METHODS

The human ovaries used in the present investigation were provided by the surgeons of the Raja Hospital, Jullundur. Small pieces were treated according to the histochemical techniques tabulated in the appendix (p. 385) to this paper. For the study of lipids the material was embedded in gelatine after Baker (1946, 1949). After Carnoy and Zenker fixations the material was embedded in paraffin.

RESULTS AND DISCUSSION

The various histochemical techniques listed in the appendix reveal in the cytoplasm of the human oocyte two categories of sudanophil lipids that are here called L_1 and L_2 for the sake of brevity. The mitochondria and yolk granules also contain sudanophil lipids.

L_1 *bodies*. These are in the form of granules and spheres. They first appear near the nucleus (fig. 1, A). With further development of the oocyte, they become larger (fig. 1, B). Later they begin to disperse (fig. 1, C) and occupy the peripheral cytoplasm in the larger oocytes (fig. 1, D). They are also present in the follicular epithelium of young oocytes (fig. 1, B, C).

The L_1 bodies consist of phospholipids and triglycerides (see appendix for the histochemical reactions). They seem to be identical with the so-called 'Golgi material' of Aykroyd (1938) and Beams and Sheehan (1941).

The L_3 bodies, which consist of triglycerides in reptilian oocytes and of triglycerides and cholesterol and its esters in those of birds, do not occur in

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the mammalian egg. The lipoprotein bodies, described in the young oocytes of birds (Guraya, 1957, 1959*b*), do not appear in those of reptiles or mammals. They have been identified as 'Golgi bodies' by some earlier workers.

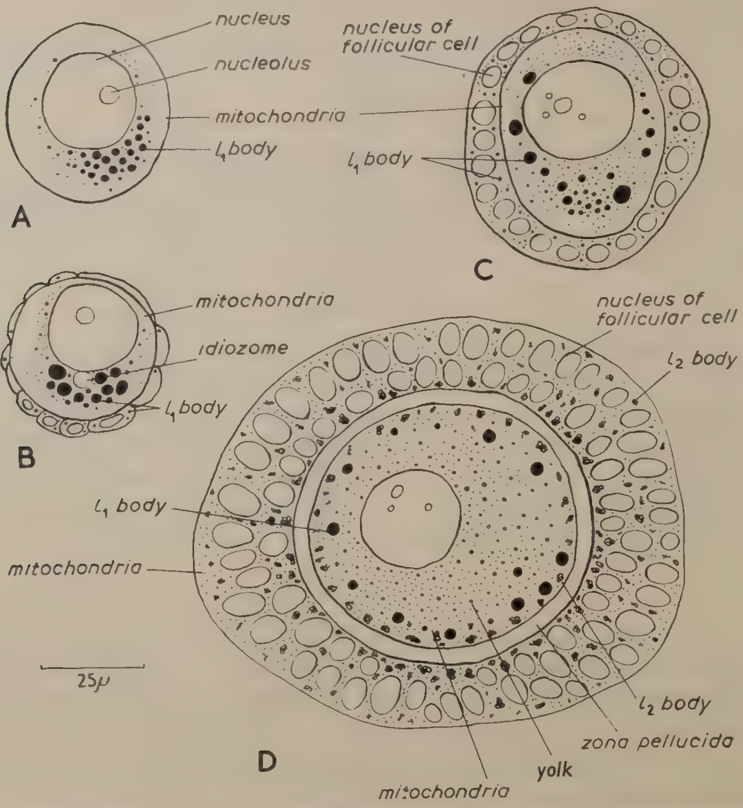

FIG. 1. Human oocytes; FCa+PC / SBB preparation. A, showing L_1 bodies and mitochondria near the nucleus of a young oocyte. B, showing L_1 bodies, mitochondria, and idiozome near the nucleus. C, showing dispersion of L_1 bodies and mitochondria; also L_1 bodies and mitochondria in the cells of the follicular epithelium. D, showing L_1 and L_2 bodies, mitochondria, and yolk granules in the cytoplasm of the larger oocyte, and L_2 bodies and mitochondria in its follicular epithelial cells.

L_2 bodies. In the human oocyte these are in the form of aggregations of granules or of fenestrated plates having sudanophobe areas. They appear at the periphery of the large oocytes, and also in the follicular epithelium surrounding these (fig. 1, D). Their sudanophil material is composed of a peculiar type of phospholipids. They were missed by Aykroyd and by Beams and Sheehan.

The L_2 bodies differ from the L_1 bodies in that they are preserved only when the material is fixed in formaldehyde / calcium and postchromed in dichromate / calcium after Baker (1946, 1956). This applies also to the L_2 bodies in the oocytes of various amniotes studied by the author. The L_2 bodies either were missed by earlier workers, or else their incompletely fixed material was identified as 'Golgi apparatus', 'Golgi material', 'Golgi bodies', 'Golgi groups', 'clusters of Golgi bodies', 'compound Golgi bodies', &c. (See previous publications of the author.) Gupta and others (1959) have described the L_2 bodies of the present author as 'fenestrated bodies' in the oocyte of the rat.

Mitochondria. The mitochondria, in the form of granules, lie near the nucleus in young oocytes (fig. 1, A, B). With the growth of the oocyte they begin to disperse (fig. 1, C), till they are distributed throughout the cytoplasm in the larger oocytes (fig. 1, D). They give reactions for phospholipids and proteins.

Yolk. The sudanophil yolk granules, which are larger than the mitochondria, are seen among the latter in the larger oocytes (fig. 1, D).

Idiozome. A sudanophobe area identical with the idiozome of Beams and Sheehan is shown in fig. 1, B. It does not occur in large oocytes.

Various lipid-containing bodies (L_1, L_2, and L_3 bodies, lipoprotein bodies, various types of yolk, mitochondria) occur in the oocytes of reptiles, birds, and mammals. Their chemical composition is diverse. When the 'Golgi' methods are applied, osmium or silver is deposited on them and they are darkened. Thus there is no particular object in these cells to which the name of Golgi can properly be attached. Baker (1957) and Nath (1957) have denied the validity of the hypothesis that all cells of animals contain a cytoplasmic inclusion homologous with the internal reticular apparatus described by Golgi in the neurones of vertebrates.

This work was carried out in the Zoology Department, Panjab University, Hoshiarpur. The author is greatly indebted to Professor Vishwa Nath for providing facilities for the work, and for his help and encouragement throughout the study. I am grateful to the surgeons of the Raja Hospital, Jullundur, for providing the human ovaries.

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APPENDIX

Table showing the histochemical reactions of cytoplasmic inclusions in human oocytes

(Gelatine sections were cut at 10 μ and paraffin at $7\frac{1}{2}$ μ)

Technique	*Fixation*	*Reference*	*L_1 bodies*	*L_2 bodies*	*Mitochondria*	*Yolk*	*Idiozome*
Sudan black B(SBB) in 70% ethanol	FCa+PC	Baker, 1946, 1956	+++	+++	++	+++	O
Sudan black B(SBB) in 70% ethanol	FCa; FS+PC; F	Baker, 1944, 1949; Pearse, 1954	+++	NF	++	+++	O
SBB in propylene glycol . .	FCa+PC	Chiffelle and Putt, 1951	+++	+++	++	+++	O
Nile blue	FCa+PC	Cain, 1947, 1948	Pk ++	O	O	O	O
Sudan III and IV . . .	FCa+PC	Kay and Whitehead, 1941; Govan, 1944	+++	++	+	++	O
Fettrot 7B	FCa+PC	Pearse, 1954	+++	++	+	++	O
Acid haematein (AH) . . .	FCa+PC	Baker, 1946	++	+++	++	++	O
AH * PE	WB+PE	Baker, 1946	O	O	O	O	O
Fischler's test	F	Pearse, 1954; Lillie, 1954	O	O	O	O	O
Schultz's cholesterol test . .	FCa+PC; FCa; FS+PC; F	Gomori, 1952; Lillie, 1954	O	O	O	O	O
Mercuric bromophenol blue . .	C; WB+PE	Mazia and others, 1953	O	O	++	+++	O
Periodic acid / Schiff (PAS) .	WB+PE; C; Z	Hotchkiss, 1948	O	O	O	++	O
SBB * acetone and ethanol . .	Fr	Pearse, 1954	O	O	O	O	O
SBB * PE	WB+PE	Baker, 1946	O	O	O	O	O

Key to lettering: * = after treatment with; C = Carnoy; F = 10% neutral formalin; FCa = formaldehyde calcium; FCa+PC = formaldehyde / calcium and postchromed; Fr = fresh material; FS+PC = formaldehyde / saline and postchromed; NF = not fixed; Pk = pink; WB+PE = weak Bouin followed by pyridine extraction; Z = Zenker; +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = negative.

Coloration of the Golgi–Nissl Network in a Vertebrate Neurone by Sudan Black

By S. K. MALHOTRA

(*From the Cytological Laboratory, Department of Zoology, Oxford*)

With one plate (fig. 1)

SUMMARY

Though it is generally considered that the cytoplasmic inclusions, commonly described as 'Golgi apparatus', contain phospholipid, the routine histochemical tests for lipid do not reveal the apparatus in the neurone of vertebrates. A technique for colouring the apparatus in the neurones of vertebrates with Sudan black is described in this paper.

IT is often assumed that the cytoplasmic inclusions generally described as Golgi apparatus contain phospholipid, which reduces osmium tetroxide or silver nitrate in the routine Golgi techniques. In the neurones of vertebrates, however, the standard histochemical tests for lipid, namely Sudan black and acid haematein, do not ordinarily reveal the characteristic 'apparatus' of Golgi (Casselman and Baker, 1955; Malhotra, 1959), even after powerful unmasking agents (Clayton, 1959) have been used in fixation (David and Brown, 1961; Malhotra, 1961).

In this paper a technique for colouring the apparatus in the neurones of vertebrates with Sudan black is described. This technique is essentially the same as one devised by Dr. O. L. Thomas (1948), who kindly sent me a preparation of a dorsal root ganglion of the kitten prepared by his method. The neurones possessed a Sudanophil inclusion very similar in appearance to the recticular apparatus of Golgi. The method used by myself is as follows:

(1) Fix in Helly's (1903) fluid for about 18 h.
(2) Postchrome in a saturated solution of potassium dichromate for about 36 h at 37° C.
(3) Wash in running water for several hours.
(4) Dehydrate and embed in paraffin.
(5) Colour the sections for ½ h in a saturated solution of Sudan black in 70% ethanol. (Thomas recommends colouring for ½ h at 60° C, but room temperature seems equally effective.)
(6) Rinse in 50% ethanol and bring sections to distilled water.
(7) Mount in Farrants's medium.

Dorsal root ganglia of the mouse were fixed and coloured with Sudan black according to this method. A reticulate object in the neurones, resembling in form and distribution the apparatus as shown by Golgi impregnation methods, is coloured with Sudan black. No such structure could be seen in these neur-

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ones when Sudan black was used on gelatine sections of dorsal root ganglia fixed in formaldehyde / calcium or formaldehyde / cadmium chloride (Malhotra, 1961).

When paraffin sections coloured with Sudan black are placed in 70% ethanol, the colour is not readily removed from the network. These observations would suggest that the colouring might possibly be due to some acidic substance and was not necessarily associated with the presence of lipid, since Sudan black is capable of acting as a weak basic dye (Baker, 1958; Casselman, 1959). Nevertheless, there is strong reason for believing that a lipid component of the network is blackened, because acetylated Sudan black, which cannot act as a dye (Casselman, 1954, 1959), is equally effective. It would thus appear that the lipid, which is ordinarily masked (probably by protein) in the object under investigation, becomes freely available when Thomas's technique is used; and it can then be demonstrated by the use of Sudan black.

It has recently been suggested (Malhotra, 1959; David and others, 1960) that the apparatus of Golgi in the neurones of vertebrates results from a deposit of silver or osmium on the basiphil reticulum of Nissl. It is therefore instructive to decolorize a Sudan black preparation that shows the typical network in the neurones and then stain the same section with a basic dye. The results of such an experiment are illustrated in fig. 1, A, B. It is clearly indicated that the same cytoplasmic inclusion is revealed by both Sudan black and basic dyes. In electron micrographs the object, corresponding to the basiphil network, is seen to be made up of membraneous endoplasmic reticulum and the small ribonucleoprotein particles associated with it (Hess, 1955; Palay and Palade, 1955; Palay, 1956; Young, 1956; Malhotra and Meek, 1960). These ribonucleoprotein particles are presumably the components that take up basic dyes (in the Nissl technique), while colouring with Sudan black is more likely to be associated with the membraneous endoplasmic reticulum, on which silver nitrate or osmium tetroxide is reduced in the Golgi techniques (Malhotra, 1959; Malhotra and Meek, 1960).

I am grateful to Dr. J. R. Baker, F.R.S., and Professor Sir A. C. Hardy, F.R.S., for their kind help. The award of a Senior Studentship under the Royal Commission for the Exhibition of 1851 is greatly appreciated. It is a pleasure to thank Dr. O. L. Thomas for communicating his unpublished work to me.

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FIG. 1 (plate). A, dorsal root ganglion cells of the mouse, prepared by fixation in Helly's fluid, postchroming, and colouring with Sudan black.

B, the same cells as illustrated in A after decolorizing and then staining with the basic dye, cresyl violet. Note that the same cytoplasmic inclusions are revealed by both Sudan black and cresyl violet; compare *a* and *a*, *b* and *b*, and so on.

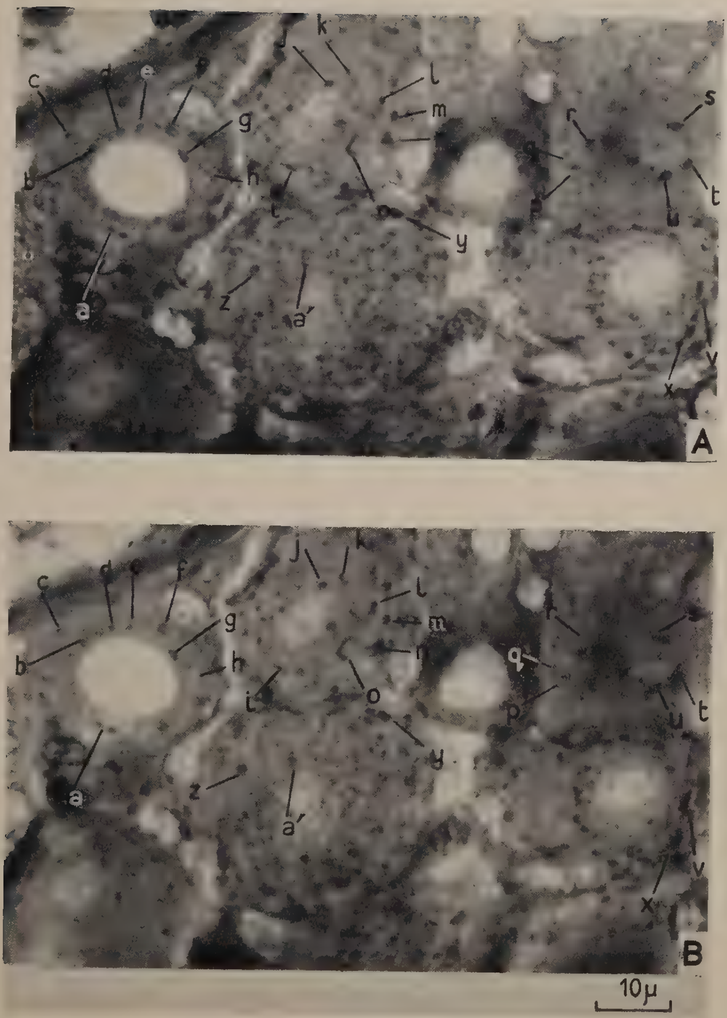

Fig. 1

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The Histochemical Recognition of Lipid in the Cytoplasmic Network of Neurones of Vertebrates

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With two plates (figs. 1 and 2)

SUMMARY

1. A method for the histochemical demonstration of the lipid of the cytoplasmic network ('Golgi apparatus', 'Nissl complex') of the neurones of vertebrates was accidentally discovered.

2. Very small pieces of tissue are fixed in Lewitsky / calcium or Flemming / calcium, postchromed, and embedded in gelatine. Frozen sections are cut at 0·5 to 5 μ, and coloured in Sudan black after bleaching.

3. The intensity of the colouring of lipid by Sudan black can be increased by centrifuging the tissue before fixation.

4. The method described in this paper demonstrates lipid in the strands and bodies of the cytoplasmic network of vertebrate neurones. It was shown, in sections subsequently decolorized and then stained in a basic dye, that the sudanophil reticular objects are the Nissl complex.

INTRODUCTION

MALHOTRA (1959, 1960) demonstrated by interference microscopy a cytoplasmic network in living vertebrate neurones, and presented evidence that this network corresponded to the Nissl complex (Nissl, 1894), the Golgi apparatus (Golgi, 1898), and the endoplasmic reticulum (Palade, 1955; Palay and Palade, 1955; Palay, 1956) of the fixed cell. Malhotra's findings have been confirmed and extended in this laboratory (David, Mallion, and Brown, 1960; David and Brown, 1961 *a*, *b*; David, Brown, and Mallion, 1961). The results of recent biochemical studies suggest that the endoplasmic reticulum of vertebrate neurones contains a complex of ribonucleic acid (RNA) conjugated with basic proteins and phospholipids (Waelsch, 1957; Clouet, 1957; Clouet and Richter, 1959). Yet proven methods for the histochemical recognition of lipid, such as colouring in Sudan black after fixation in formaldehyde / calcium, generally demonstrate only small separate globules in the cytoplasm of vertebrate neurones (Baker, 1944, 1949, 1957 *a*, *b*, 1959; Thomas, 1948; Casselman and Baker, 1955; Malhotra, 1959; David and Brown, 1961 *a*). In the course of preparing vertebrate neurones for other work David and Brown, 1961 *a*, *b*; David, Brown, and Mallion, 1961), we found hat the lipid of the cytoplasmic network could be demonstrated when the neurones were fixed in two new reagents; this lipid could be coloured in Sudan black quite strongly if 'unmasked' in a certain way.

This paper is restricted to the description of the improved methods of

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fixation and 'unmasking' which enabled us to recognize the presence of lipid in the network by the use of Sudan black: the detailed characterization of this lipid will be considered in another paper.

MATERIALS

The neurones of the dorsal root ganglia of immature cats and rabbits were used. There is no reason to suppose that the results would have been different had other vertebrate neurones been chosen.

METHODS

Fixation

The original aim of the experiments on fixation was to improve the preservation of the form of lipid globules. Baker (1944, 1958*a*) and Chou and Meek (1958) showed that the addition of calcium ions to certain fixatives rendered them less liable to extract lipid and to distort cytoplasmic inclusions containing lipid. Baker (1956) also introduced a modified Flemming solution, Lewitsky / saline or LS. This does not contain calcium ions, but is a superb cytological fixative. It occurred to us that calcium ions might be beneficial in LS as well, and made this up to contain 1% w/v anhydrous calcium chloride instead of the 0·75% sodium chloride of the original formula. We call this fluid Lewitsky / calcium or L/Ca.

L/Ca, though an excellent fixative for lipids and proteins (see fig. 1), does not fix RNA adequately. In studies of the cytoplasmic network of vertebrate neurones, in which we may wish to demonstrate the Nissl complex by the use of basic dyes in the same piece used for the demonstration of lipids, this is a serious drawback. For this reason we tried adding 1% anhydrous calcium chloride to Flemming's original fluid (Flemming, 1882), which contains 5% acetic acid and therefore can be expected to fix RNA adequately. We call this modified fluid Flemming / calcium or Fl/Ca.

Since the constituents of L/Ca and Fl/Ca penetrate tissues at different rates, it is important that the pieces to be fixed should be as small as possible. Tissues should be fixed overnight (15–18 h), and washed in running water for 5 to 6 h.

After-treatments

Postchroming should be carried out exactly as in the acid haematein technique (Baker, 1946). Lipid is sufficiently well fixed by L/Ca and Fl/Ca, with postchroming, not to be extracted by the after-treatments required to produce paraffin sections. But, since the tissue then becomes severely shrunken and distorted, embedding in gelatine is preferable. Baker's (1944) method gives excellent results.

The cytoplasm of vertebrate neurones is crowded with inclusions of various kinds. For this reason it is best to cut extremely thin frozen sections. It is not difficult to cut 3 μ frozen sections with an ordinary sledge microtome. We use a Sartorius no. 31a microtome, equipped with a knife having a cutting bevel of

35°. Thinner sections, down to 0·5 μ, are cut with a Cambridge rocker microtome in a cryostat at −20° C.

Before treating sections of tissues fixed in L/Ca or Fl/Ca with colouring agents, it is necessary to oxidize away the osmium combined with the tissue. A mixture containing 99 ml of 1% w/v aq. potassium permanganate and 1 ml of concentrated sulphuric acid bleaches sections in 30 min. The permanganate is then removed in 2% w/v aq. oxalic acid, and the sections are washed in running water for 30 min.

Colouring and dyeing methods

Lipids of all kinds are best demonstrated by Baker's standard Sudan black (Baker, 1944, 1949). We colour for 10 min at 60° C. Control sections are subsequently extracted in 90% v/v ethanol for 15 min (Baker, 1958*b*). Berg's very sensitive method of fluorochroming lipids in 3:4-benzpyrene (Berg, 1951) could not be used, since the osmium remaining in the tissue after the permanganate bleach effectively quenches the induced fluorescence. The acid haematein test for phospholipids (Baker, 1946) can be used satisfactorily (David and Brown, 1961*b*).

The Nissl complex is best demonstrated by dyeing in cresyl violet monomer (David, 1955). This method was applied directly, and to sections that had been coloured in Sudan black, with subsequent removal of the lysochrome in 90% ethanol. Other methods of dyeing the Nissl complex, such as gallocyanine / chrome alum (Einarson, 1932, 1951), gave disappointing results.

Method of unmasking lipid

A number of unfixed dorsal root ganglia were ultra-centrifuged in order to stratify the contents of the neurones (David and Brown, 1961*a*; David, Brown, and Mallion, 1961). The contents of neurones resist stratification to a surprising extent: living neurones centrifuged at 33,000 *g* for 30 min could not be distinguished from untreated neurones by interference microscopy. Some of the centrifuged neurones were subsequently fixed in L/Ca or Fl/Ca, and coloured in Sudan black. Much to our surprise, the cytoplasmic network now coloured much more vigorously than in untreated neurones. The network remains strongly sudanophil after stratification at 95,000 *g*. The following procedure is recommended for increasing the sudanophilia of the network.

Suspend small fragments of dorsal root ganglia in about 10 ml of ice-cold Baker's (1944), Ringer's, or Krebs's saline. Transfer the suspension to a chilled centrifuge-tube, containing a 1 cm layer of 30% w/v bovine plasma albumin (either crystallized or 'fraction V') dissolved in the same saline. Avoid stirring up the albumin. The tissue should settle at the interface between the two liquids, where a density-gradient gradually forms by diffusion of the albumin. The albumin prevents the crushing of the neurones against the bottom of the tube during centrifugation. Centrifuge at 33,000 *g* for 30 min at 2° C. Then fix in L/Ca or Fl/Ca.

RESULTS

Neurones fixed in L/Ca after centrifugation are illustrated in fig. 1. The form of the neurones and the cytoplasmic inclusions are faithfully preserved. Centrifuged neurones fixed in Fl/Ca are shown in fig. 2.

A sudanophil cytoplasmic network, similar in form to that described by Malhotra (1959), can be seen in neurones fixed in either reagent (figs. 1; 2, A). It is composed of irregular bodies and thin strands. Some of the bodies are small and nearly spherical (figs. 1, A; 2, A); others are massive objects with rather indistinct edges, as much as 8 μ long and about 2 to 4 μ wide (fig. 1, B). The strands are about 0·5 μ in diameter, and are peppered with minute granules that are too small to be measured accurately. The strands are associated with the bodies in such a way that the bodies give the impression of being enlarged nodal points in a three-dimensional network. Sudanophobe elongate spaces, about 2 to 5 μ long and 1 to 3 μ wide, are sometimes seen between adjacent strands.

Both fixatives dissolve away a large proportion of the nuclear contents. The plasmosome and chromatin clumps within the nucleus are sudanophil. The general preservation of form is rather better after fixation in L/Ca than in Fl/Ca. However, the cytoplasmic network stands out more sharply in neurones fixed in Fl/Ca, and it can be stained adequately in basic dyes (fig. 2, B). The results are qualitatively the same in neurones that are fixed without having been centrifuged, but then the cytoplasmic network and other inclusions are much less sudanophil.

In view of the consistent finding by previous workers that the cytoplasmic network of vertebrate neurones gives negative results to tests for the histochemical recognition of lipid (for references see p. 391), it is necessary to provide clear evidence that Sudan black acted as a lysochrome for the lipid of the network and not as a weak basic dye. Sections coloured in Sudan black were immersed in 90% ethanol for 15 min; this solvent extracted all the colour from the cytoplasmic network. This suggests that the Sudan black must have coloured the network by lysochromy (Baker, 1958*b*). Other pieces of dorsal root ganglia were extracted in pyridine at 60° C for 24 h, after fixation in weak Bouin (Baker, 1946). In neurones from these ganglia it was not possible to colour the cytoplasmic network in Sudan black. The findings of these control-experiments leave no doubt that Sudan black coloured the lipid of the cytoplasmic network of the neurones fixed in L/Ca and Fl/Ca (see figs. 1; 2, A). These findings will be discussed in greater detail in a later publication.

In view of the immense number and variety of the cytoplasmic inclusions of the neurones of dorsal root ganglia, it is necessary to provide direct evidence that the sudanophil network is the Nissl complex, that is to say the network that stains in basic dyes. Neurones coloured in Sudan black were mounted in

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FIG. 1 (plate). Neurones of the dorsal root ganglia of the cat, fixed in L/Ca after slight centrifugation, coloured in Sudan black and photographed in monochromatic light of 600 mμ. A is a neurone in a 3-μ gelatine section, and B in a thinner section (about 1 μ).

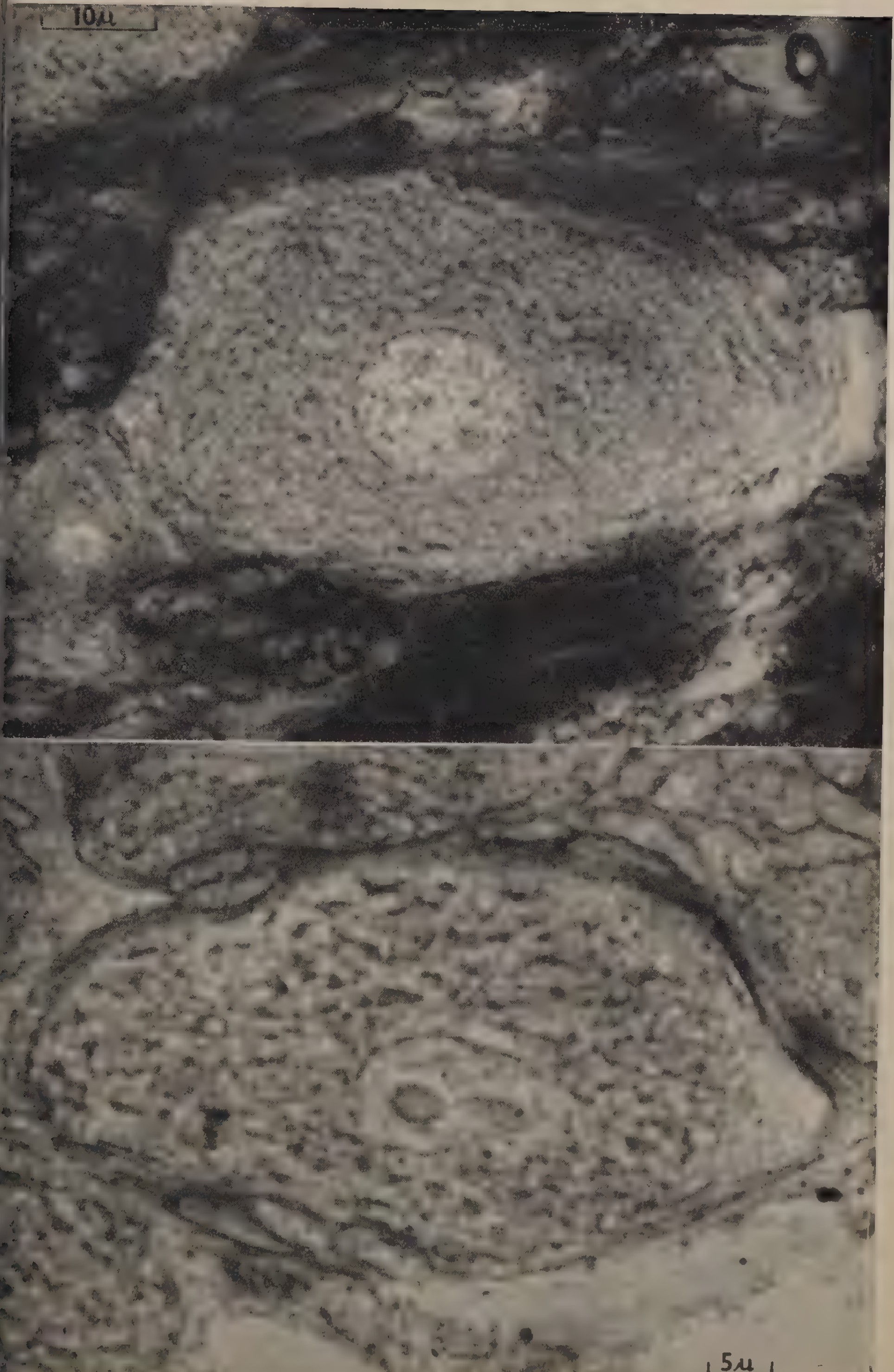

FIG. 1

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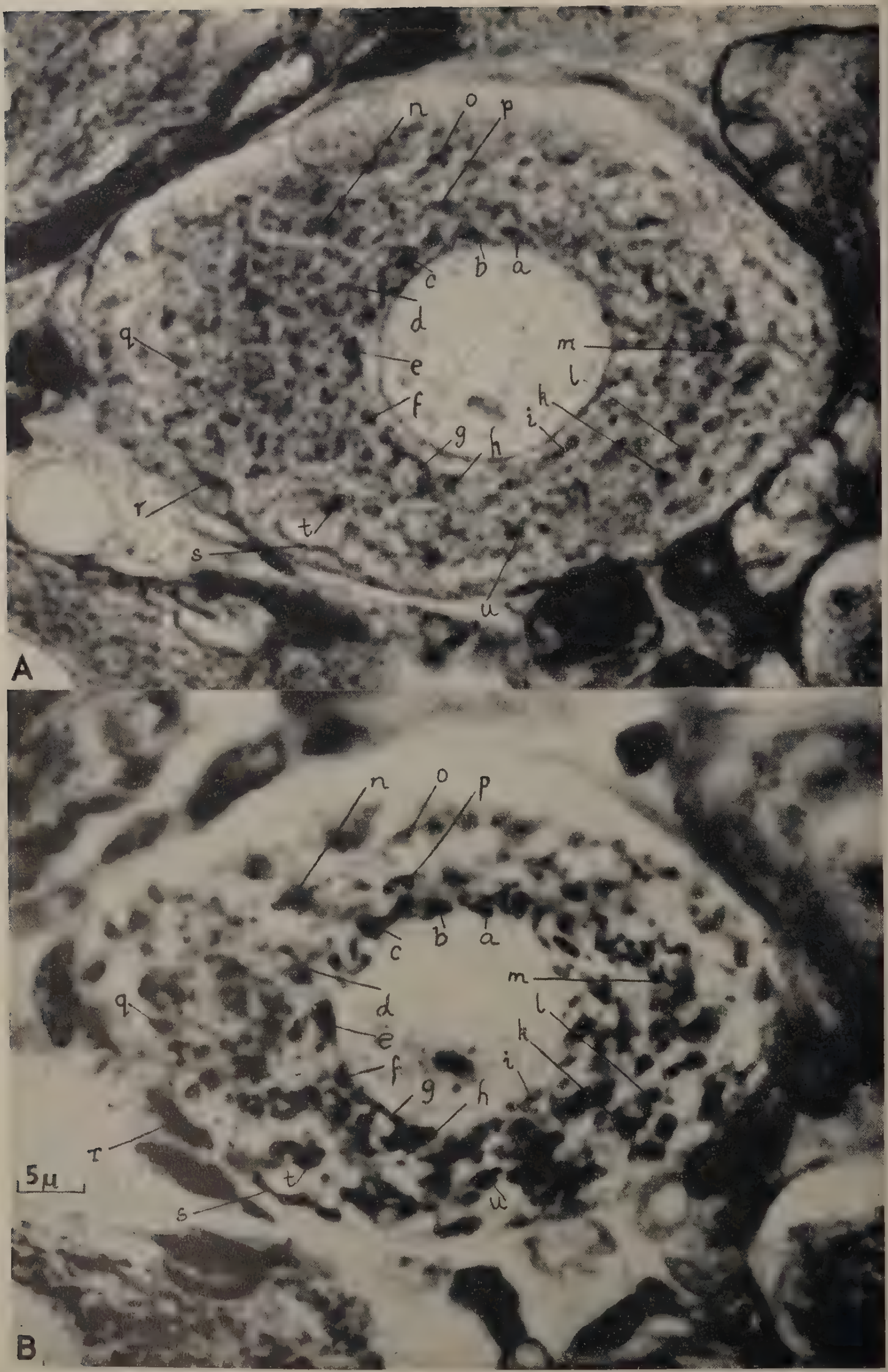

FIG. 2

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anhydrous glycerol, and photographed (fig. 2, A). Then, the Sudan black was completely extracted in 90% ethanol, and the sections were dyed in cresyl violet. The same neurones were found and photographed again (fig. 2, B). The sudanophil network is also basiphil; therefore, it is the Nissl complex. This is not to say that all sudanophil objects are part of the Nissl complex: lipid globules are not, though they are strongly sudanophil.

DISCUSSION

It is not clear why the addition of calcium ions to modified Flemming solutions should facilitate the colouring of the lipid of the cytoplasmic network. Calcium ions prevent the emulsification of lipids, but one would not have thought that the failure of previous techniques to show lipid in the network was due to lipid being removed. Formaldehyde / calcium (Baker, 1944), as used for the histochemical study of lipid, extracts some glycerophosphate (Chayen, Gahan, and LaCour, 1959); but LS does not. Yet the cytoplasmic network is sudanophobe after fixation in either formaldehyde / calcium or LS. Is there any reason to suppose that LS allows the escape of lipids that are fixed by L/Ca? The action of calcium ions in Fl/Ca is even more obscure. 'Indifferent' salts appear to have little or no effect when used in coagulating fixatives that contain much acetic acid (Baker, 1958*b*).

It is not immediately obvious why centrifugation should have any effect on the ease with which lipid is coloured by Sudan black. It is true that during centrifugation the neurones were subjected to a pressure of between 3,000 and 13,000 lb/sq. in. Is it conceivable that some of the bonds between lipids and proteins or RNA are sheared by these pressures? On the other hand, the lipid may have been masked by a small quantity of a substance of either higher or lower specific gravity than that of the cell as a whole; this substance might have been displaced by centrifugation. We have not detected the accumulation of a substance at either pole of the cell after centrifugation adequate to render the network sudanophil, but not to stratify it. But, if this substance were present in very small amounts, it might easily be overlooked. Further experiments are planned to clarify these problems.

After the work described in this paper was completed, we heard that Prof. O. L. Thomas and Dr. S. K. Malhotra had independently succeeded in demonstrating histochemically the lipid of the cytoplasmic network of vertebrate neurones by a different method.

FIG. 2 (plate). These are two photographs of the same dorsal root neurone, fixed in Fl/Ca after slight centrifugation, embedded in gelatine, and cut at 3 μ.

A, coloured in Sudan black, mounted in glycerol, and photographed at 600 mμ, to demonstrate lipid.

B, dyed in cresyl violet and photographed at 546 mμ after extraction of the lysochrome, to show that the sudanophil network is the Nissl complex.

The small letters indicate structures that are visible in both photographs. Compare *a* with *a*, *b* with *b*, and so on. Since extraction of the lysochrome and dyeing produce shrinkage and distortion, it is not possible to obtain a perfect correspondence of all parts of a cell by this method.

We are grateful to Dr. D. Richter, Director of this Research Unit, for his continued support of our cytological work. It is a pleasure to acknowledge the help we have received on many occasions from Dr. John R. Baker, F.R.S., and Dr. S. K. Malhotra, at the Cytological Laboratory of the Department of Zoology, Oxford University.

APPENDIX

The formulae given below will be found convenient for the preparation of L/Ca and Fl/Ca. The stock solutions are quite stable, as is L/Ca; Fl/Ca deteriorates fairly rapidly.

	Working solutions	
Stock solutions	*L/Ca*	*Fl/Ca*
	(ml)	(ml)
Osmium tetroxide, 2% w/v aq.	2·0	2·0
Chromium trioxide, 5% w/v aq.	1·5	1·5
Acetic acid, 20% v/v aq.	—	2·5
Anhydrous calcium chloride, 10% w/v aq.	1·0	1·0
Distilled or deionized water	5·5	3·0
	10·0	10·0

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The Cytology and Histochemistry of the Neurones of *Periplaneta americana*

By DOREEN E. ASHHURST

(*From the Department of Zoology, The University, Manchester*)

With one plate (fig. 1)

SUMMARY

The only cytoplasmic inclusions visible in the neurones by the light microscope are ne lipochondria and mitochondria. It is suggested that the Golgi bodies, seen in reparations made by the Golgi techniques, are produced by the deposition of osmium r silver on the surface of the lipochondria.

The lipochondria consist of phospholipids and cerebrosides. There is also some pid in the cytoplasm, together with carbohydrates, proteins, and RNA.

INTRODUCTION

THE neurones of insects have been the subject of innumerable cytological studies. The cell inclusions which have attracted the most atten- on are the Golgi bodies. In invertebrate neurones these are of different hapes—circles, crescents, and rods—which are collectively referred to as dictyosomes'. A controversial question arises as to whether the dictyosomes re present in the living cell or are artifacts produced by the fixation and mpregnation techniques used to demonstrate them. Recent studies of insect eurones have fallen into two groups, those supporting the theory of the alidity of the Golgi bodies as true cytoplasmic inclusions (Gresson, Thread- old, and Stinson, 1956) and those presenting evidence to support the theory hat the dictyosomes are artifacts (Shafiq, 1953; Nath, 1957). The use of the ectron microscope in the study of insect neurones has further complicated his issue, since a structure seen in electron micrographs has been identified s a Golgi body (Hess, 1958).

The cockroaches, *Periplaneta americana* and *Blatta orientalis*, have been used efore for studies of insect neurones. These include the cytological work of ialkowska and Kulikowska (1919) and Muliyil (1935) and the electron icroscope studies of Hess (1958), but there appears to be no record of a istochemical study. It was, therefore, decided to undertake a histochemical udy of the neurones of *P. americana* and to consider the status of the Golgi odies in the light of the histochemical evidence.

METHODS

The neurones studied here were those of the meso- and meta-thoracic nglia. For vital staining, the ganglia were teased apart to free the neurones, hich were then stained in dilute solutions of either neutral red or Janus

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green. Stock solutions of these dyes (0·5% aqueous) were diluted as follows: neutral red, 2 drops of stock solution to 2 ml of insect Ringer; Janus green, 1 drop of stock solution to 5 ml of insect Ringer. The neurones were stained for about 10 min and then examined.

To demonstrate the Golgi bodies, preparations were made by the standard Golgi techniques, Aoyama's silver method (Aoyama, 1929), Kolatchev's technique (Kolatchev, 1916), and the Mann–Kopsch technique (Weigl, 1910). The mitochondria were studied in preparations made by Baker's (1957*a*) 'HPO' technique. A number of preparations of the neurones were made by modifications of Bodian's protargol method (McClung Jones, 1950; Power, 1943). The histochemical tests performed on the neurones are given in the appendix.

RESULTS

The neurones lie around the periphery of the central mass of nerve-fibres in the ganglion, forming an incomplete layer between the fibres and the connective-tissue sheath. There are two main types of neurone. Some are very large, having a diameter between 30 and 55 μ, while others are much smaller, being of diameter between 14 and 30 μ. Apart from the difference in size, the two types of neurone appear to be identical.

The most characteristic structures in invertebrate neurones are the Golgi bodies or dictyosomes (fig. 1, A). In the cockroach, these structures were seen most clearly after Kolatchev's technique, but were also visible after both the Aoyama and Mann–Kopsch methods. They appear in sections as crescent-shaped bodies, but in a few instances they form complete circles; these structures are between 1·5 and 3 μ in diameter. The interior of the dictyosome is darker than the surrounding cytoplasm. It must be mentioned that the dictyosomes are visible only after the techniques listed above and that these techniques are somewhat suspect since they are dependent upon the deposition of silver or osmium, a process which may be non-specific (Baker, 1957*b*).

The question now arises whether the crescents and rings represent structures of this shape in the cell, or are a form of artifact due to the deposition of osmium or silver on some other cell inclusion. In the neurones of some other invertebrates, e.g. *Helix aspersa* and *Locusta migratoria*, it has been shown that the Golgi bodies are formed by such deposition on the lipochondria (Chou, 1957; Shafiq, 1953). In an attempt to answer this question, the neurones were examined after vital staining, and also in preparations designed to show the presence of lipids. When the neurones are vitally stained with neutral red, the cell appears to be full of small, homogeneous, spherical bodies, varying between 0·5 and 2 μ in diameter. These correspond very closely with the distribution and size of the lipochondria seen in gelatin sections coloured with Sudan black (fig. 1, C), and also with the distribution

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FIG. 1 (plate). A, section of a neurone showing the dictyosomes. Kolatchev preparation.

B, section of a neurone showing the structures coloured with Sudan black after Champy fixation.

C, section of a neurone showing the lipochondria coloured with Sudan black.

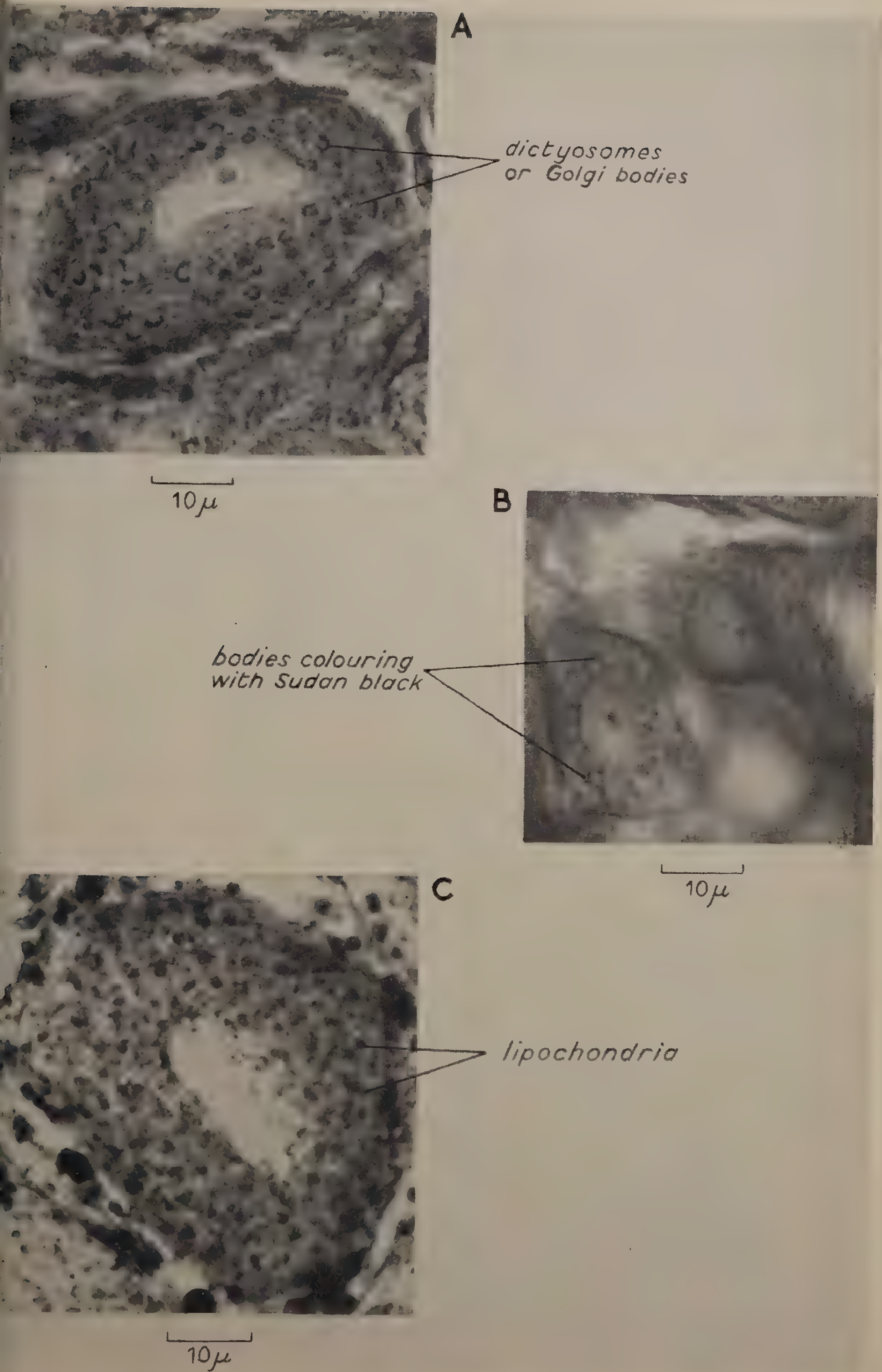
A
dictyosomes or Golgi bodies
10 μ
B
bodies colouring with Sudan black
10 μ
C
lipochondria
10 μ

Fig. 1

D. E. ASHHURST

of the Golgi bodies, but the dictyosomes are sometimes 2·5 μ across, whereas the largest lipochondria are about 2 μ in diameter. Nevertheless, these results suggest that, as in *Helix* and *Locusta*, the dictyosomes may be formed by the deposition of osmium or silver on the surface of the lipochondria, the lipids inside being removed by the subsequent dehydrating and embedding procedures.

To obtain further evidence for this suggestion ganglia were fixed in Champy's fluid, as in a Kolatchev preparation, and embedded in gelatin. Sections of the ganglia were bleached in hydrogen peroxide (20 vol. diluted 1 in 4 with water) for 2 h to remove the dark coloration due to the osmium contained in the fixative, and then coloured with Sudan black; coloration at room temperature for 30 min was found to be the most satisfactory. This procedure revealed structures which are almost indistinguishable from the Golgi bodies (fig. 1, B). They have the appearance of a dark ring or crescent, with a less dense area inside. This result provides direct evidence that the osmium tetroxide used in making Golgi preparations is reduced by the lipids in the lipochondria. The crescent shapes are most probably due to an uneven deposition of osmium, and their greater diameter compared with the lipochondria may be caused by a spherical layer of osmium around a lipochondrion breaking as the tissue was dehydrated, or because the osmium is deposited on the outside of the lipochondrion, the internal diameter being that of the globule. It therefore seems reasonable to conclude that the dictyosomes are artifacts produced by the deposition of osmium or silver on the surface of the lipochondria.

The mitochondria of the neurones can be seen in cells vitally stained with Janus green and in preparations made by Baker's HPO technique. There is a large number of mitochondria scattered throughout the neurone. Some are small granules of about 0·5 μ diameter, while others are thin rods between 1·5 and 2·5 μ long. Some of the rods appear to have a granule at each end.

Neurofibrillae in the neurones of *Periplaneta* have been described by several authors (Hess, 1958; Pipa, Cook, and Richards, 1959), but they were not visible in the Bodian's protargol preparations made in this investigation. This result may be due to the origin of the protargol (G. T. Gurr), since some authorities suggest that satisfactory results are obtained only with protargol from one source (Gatenby and Beame, 1950).

The nuclei of the neurones are large, varying in diameter between 9 and 20 μ. They are roughly spherical and possess an obvious nucleolus. It will be noticed that the range in nuclear size is much smaller than the range in cell size. This results in the division of the neurones into two types, the small neurones with a relatively large nucleus and little cytoplasm, and large neurones with a nucleus of similar size, but a large amount of cytoplasm.

The cell membranes of the large neurones are indented, so that processes of the surrounding connective tissue and glial cells appear to penetrate the cytoplasm of the neurones. This phenomenon has been described by Hess (1958) in his electron microscope study of the cockroach (*P. americana*) and by Wigglesworth (1959) in *Rhodnius prolixus*.

Histochemistry of the neurones

Carbohydrates. The neurones are only very weakly positive to the periodic acid / Schiff reaction; the individual cytoplasmic inclusions cannot be seen. The reaction was not increased when fixatives containing acridine and cetylpyridinium chloride, as recommended by Williams and Jackson (1956) for the preservation of mucopolysaccharides, were used. It is unaltered if the sections are first incubated in either diastase (0·1% aqueous) or saliva for $1\frac{1}{4}$ h at 37° C; this suggests that little if any glycogen is present. This result is further supported by the fact that Best's carmine test for glycogen is negative in the neurones.

Lipids. Histochemically, the lipochondria are the most complex constituent of the neurones. As mentioned previously, they can be coloured by Sudan black and also by Sudan IV. Some of their lipid is in the form of phospholipid since they are positive with Baker's acid haematein test.

The possibility exists that the lipochondria may also contain cerebrosides. Some ganglia were extracted in hot and others in cold acetone, and embedded in gelatin. The sections obtained were coloured with Sudan black. After hot acetone extraction no appreciable amount of lipid remains in the cell, but after cold acetone extraction the lipochondria are still visible although it is apparent that some lipid has been displaced into the cytoplasm. Phospholipids are not extracted by cold acetone, but cerebrosides are removed (Casselman and Baker, 1955). It appears, therefore, that the lipids removed by the cold acetone are cerebrosides, the phospholipids remaining in the lipochondria.

Of the fixatives suggested for 'unmasking' lipids (Bradbury and Clayton, 1958; Clayton, 1959), Flemming's fluid and mercuric chloride were used. But the subsequent colouring of the sections with Sudan black failed to reveal any 'masked' lipid in the neurones.

Proteins. The cytoplasmic inclusions could not be distinguished in tests for proteins (Barnard and Danielli's test, Sakaguchi, Hg / nitrite).

Nucleic acids. The chromatin of the nuclei gives a positive reaction with the Feulgen test. The cytoplasm is strongly basiphil; this was shown by the pyronin / methyl green test. This is probably due largely to the presence of RNA, since treatment with ribonuclease reduces the basiphilia considerably.

Phosphatases. Danielli's (1953) modification of Gomori's glycerophosphate technique for alkaline phosphatase was performed on sections of frozen-dried ganglia. No alkaline phosphatases could be detected in the neurones, even after the addition of disodium phosphate to the incubating mixture (a modification suggested for use when only minute quantities of enzyme are suspected to be present).

DISCUSSION

The lipochondria are shown clearly after vital staining with neutral red, or after the coloration of fixed material with Sudan black. Bodies staining with neutral red are described in the neurones of *P. americana* by Muliyil (1935), who considers that they are different from the Golgi bodies, although both go towards the centripetal pole when the cells are centrifuged. It appears probable that the neutral red bodies of Muliyil are in fact the lipochondria.

The Golgi bodies of *B. orientalis* and *P. americana* have been described previously by Bialkowska and Kulikowska (1912) and by Muliyil (1935) respectively. Their descriptions are essentially similar to the one in this paper. But they did not describe the lipochondria, and therefore the similarity in the distribution of the lipochondria and Golgi bodies within the cells was not apparent to them. This similarity of distribution suggests that the lipochondria and Golgi bodies may well be the same cell inclusions, the dictyosomes being formed as a result of the action of the Golgi techniques on the lipochondria. Further evidence for the lipid nature of the Golgi bodies is provided by their coloration with Sudan black after fixation in Champy's fluid. The main objection to this idea is that the dictyosomes are larger than the lipochondria, but this is to be expected if the osmium or silver is deposited on the outside of the lipochondrion. The crescents and rods are the result of the unevenness of the deposition of osmium and silver.

Histochemically, cockroach neurones are similar to locust neurones (Shafiq and Casselman, 1954). In both the lipochondria are all identical and may be termed 'cerephos globules' as they contain cerebroside and phospholipid (Baker, 1957*b*).

The cell inclusions seen by the use of the light microscope are also evident in electron micrographs of sections of the cockroach neurone, but in addition large numbers of small granules are found; these probably represent the basiphil material of the cytoplasm. A number of Golgi bodies are also seen in electron micrographs (Hess, 1958). These Golgi bodies are similar to those described by Gatenby, Tahmisian, Devine, and Beams (1958) in other orthopteran cells. But it has been suggested that the Golgi bodies in *Periplaneta* are artifacts produced by the deposition of osmium or silver on the lipochondria. Perhaps this can be reconciled with the electron microscope evidence; for it has been shown that the lipid globules in *H. aspersa*, which contain only phospholipid, appear in electron micrographs of material fixed in buffered osmium tetroxide as a series of curved or flat parallel lamellae with a number of scattered vesicles. If, however, calcium chloride is added to the fixative, the globules appear spherical, with concentric parallel lamellae round the periphery (Chou and Meek, 1958). It has been suggested by Schmidt (1939) that phospholipid molecules lie with their hydrophobe ends together, while the hydrophil ends associate with water. Thus in lipid globules these would form concentric layers of phospholipid and water. Baker (1958) suggests that these concentric layers remain intact during fixation if the molecular bonds are stabilized by the presence of calcium ions in the fixative, but under normal conditions of osmium fixation the layers break, with the result that a series of parallel crescent-shaped lamellae are formed. The lipochondria of *Periplaneta* contain phospholipid; so it is possible that the typical Golgi bodies seen in the electron micrographs could be produced in this way by the action of the osmium fixative on the phospholipids in the lipochondria. If this is so, then the discrepancy between the results with the light microscope described in this paper, and those with the electron microscope, may be apparent rather than real.

I should like to thank Professor R. Dennell for his suggestions while this paper was in preparation and Mr. P. Howarth for preparing the photomicrographs. I am grateful to Professor H. Graham Cannon, F.R.S., for his encouragement and interest in this work.

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APPENDIX

Table of methods and results

Test	Reference	*Neurones*	
		Cytoplasm	*Lipochondria*
Carbohydrates			
PAS	Pearse, 1960	+	not visible
PAS with no oxidation		O	,,
PAS after diastase		+	,,
PAS after saliva		+	,,
Best's carmine	Casselman, 1959	O	,,
Lipids			
Sudan IV	Herxheimer, 1901	+	++
Sudan black	Baker, 1945, 1949, 1956*b*	+	++
Sudan black after cold acetone	Casselman and Baker, 1955	+	+
Sudan black after hot acetone	Casselman and Baker, 1955	O	O
Acid haematein	Baker, 1946	O	++
Acid haematein: pyridine extraction	Baker, 1946	O	O
Nile blue	Cain, 1947	blue	blue
Liebermann	Lison, 1953	O	O
Windaus	Lison, 1953	O	O
Proteins, &c.			
Coupling reaction	Barnard and Danielli, 1956	++	not visible
Coupling reaction after benzoylation	Barnard and Danielli, 1956	O	,,
Sakaguchi	Baker, 1947	+	,,
Hg / nitrite	Baker, 1956*a*	+	,,
Nucleic acids			
Feulgen	Feulgen and Rossenbeck, 1924	chromatin+	O
Feulgen control		O	O
Pyronin / methyl green	Jordan and Baker, 1955	++	not visible
Pyronin / methyl green after RNAase	Bradbury, 1956	O	,,
Phosphatase			
Gomori's test for alkaline phosphatase	Danielli, 1953	O	,,

KEY: ++ = medium reaction; + = weak reaction; O = negative reaction.

Tyrosinase Activity in the Pigmented Cells of the Nucleus Substantiae Nigrae. I. Monophenolase and Diphenolase Activity

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With one plate (fig. 1)

SUMMARY

The method of formation of black pigment in cells of the nucleus substantiae nigrae has been investigated by employing histochemical techniques which detect tyrosinase, the enzyme responsible for melanin formation in skin melanocytes. Frozen sections of the mid-brains of adult monkeys and cats were incubated in L-tyrosine, to detect the monophenolase activity of tyrosinase; other sections were incubated in L-dopa to detect the diphenolase (dopa oxidase) activity of the enzyme. The dopa, but not the tyrosine, was converted into melanin by the cells of the nucleus substantiae nigrae, resulting in blackening of the cytoplasm.

The intensity of blackening of nigra cells resulting from incubation in L-dopa depended on physical factors. The optimum pH of the reaction ranged between 6·8 and 7·4; the optimum temperature was 37° C; the concentration of substrate employed and the duration of incubation were additional factors which influenced the intensity of the reaction. The conversion of dopa to melanin by nigra cells was prevented by general enzyme inhibitors and by specific inhibitors of tyrosinase, which confirmed the specificity of the reaction for the diphenolase activity of tyrosinase.

The significance of the presence of diphenolase activity, in the absence of monophenolase activity, in adult nigra cells has been discussed in relation to pigment formation in the brain.

INTRODUCTION

PIGMENT granules are present in the cells of certain nuclei of the adult human brain-stem, especially in the nucleus substantiae nigrae and the nucleus locus coeruleus. They have also been demonstrated in the nucleus substantiae nigrae of many mammals (Marsden, 1960). The significance of this pigment in the brain and the biochemical processes involved in its formation are unknown. The brown or black intracytoplasmic granules are usually considered to be melanin, and resemble melanin granules in skin melanocytes in many of their physical and chemical properties (Lillie, 1954). Pigment in the brain may be formed by processes similar to those producing melanin in the skin melanocytes. The chemical reactions occurring in the formation of melanin in the skin were first established by Raper (1928) and have subsequently been confirmed (Lerner, 1953).

Cutaneous melanin is formed by the catalytic action of tyrosinase, an

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enzyme present in melanocytes, on the amino-acid tyrosine. Tyrosinase catalyses the oxidation of tyrosine to 3,4-dihydroxyphenylalanine (dopa), and the subsequent oxidation of dopa to dopa-quinone. Dopa-quinone is subsequently converted through intermediate stages to 5,6-dihydroxyindole, which finally polymerizes to form melanin. Tyrosinase, which is a member of the copper-containing group of oxidases termed phenolases (Mason, 1956), exhibits a monophenolase and a diphenolase activity. The monophenolase activity catalyses the oxidation of tyrosine to dopa, and the diphenolase (dopa oxidase) activity catalyses the oxidation of dopa to dopa-quinone (Lerner and Fitzpatrick, 1950), which is subsequently converted to melanin non-enzymatically. Both of these activities of the one enzyme are essential in the production of cutaneous melanin.

The monophenolase activity of tyrosinase in the skin may be detected histochemically by incubating fresh tissue in L-tyrosine. In the presence of monophenolase activity the tyrosine is converted into melanin with blackening of the cytoplasm of cells containing the enzyme (Fitzpatrick, Becker, Lerner, and Montgomery, 1950). The diphenolase activity of tyrosinase may be detected by using dopa as the substrate; this results in a similar blackening of the cytoplasm of cells containing the enzyme, owing to the formation of melanin from dopa (Bloch, 1916). The similarity of pigment in the brain-stem to cutaneous melanin has suggested that it may also be formed by the action of tyrosinase on tyrosine, and therefore the histochemical techniques for demonstrating the two activities of the enzyme in the skin have been applied to the pigmented cells of the nucleus substantiae nigrae.

Material and Methods

The monkey and the cat both possess pigmented nigra cells (Scherer, 1939; Brown, 1943) and were used for this study. Under conventional light microscopy, such as was used in this study, the pigment was not visible in unstained preparations. Therefore additional pigment formed as a result of monophenolase or diphenolase activity would be immediately apparent. The brains of 4 adult Rhesus monkeys (*Macaca rhesus*) and 5 adult cats (*Felis felis*) were removed immediately after death, and fixed in 10% neutral formalin for 1 h. The mid-brain was then removed and fixed for a further 2 h in the same solution. Frozen transverse sections of the mid-brain, 10 to 15 μ thick, were mounted on clean glass slides and dried in air to ensure adhesion. These sections were either used immediately or stored in a deep-freeze at −20° C for up to 3 weeks.

Reaction for monophenolase activity

A modification of the method of Fitzpatrick, Becker, Lerner, and Montgomery (1950) was employed. Sections were incubated for 24, 36, and 48 h at 37° C in 1 : 1000 L-tyrosine in 0·1 M phosphate buffer at pH 6·8. Sections incubated in buffer solutions alone were used as controls.

Reaction for diphenolase activity

A modification of the method of Bloch (1916) was employed. Sections were incubated for 24 h at 37° C in 1:1000 L-dopa in 0·1 M phosphate buffer at pH 6·8. Sections incubated in buffer solutions alone were used as controls.

The effects of variations of the following physical factors on the reaction for diphenolase activity were independently investigated:

pH. Sections were incubated for 24 h at 37° C in 1:1000 L-dopa buffered at pH 5·0, 6·0, 6·8, 7·4, and 8·0.

Substrate concentration. Sections were incubated for 24 h at 37° C in 1:1000, 2:1000, and 3:1000 concentrations of L-dopa buffered at pH 6·8.

Duration of incubation. Sections were incubated for 2, 6, 12, 18, 24, 48, and 56 h at 37° C in 1:1000 L-dopa buffered at pH 6·8.

Temperature. Sections were incubated for 24 h at 4° C, 18° C, 37° C, and 60° C in 1:1000 L-dopa buffered at pH 6·8.

The specificity of the diphenolase reaction was investigated by using a series of inhibitors of melanin formation. Lerner and Fitzpatrick (1950) and Lorincz (1954) described a number of groups of inhibitors of cutaneous melanogenesis on the basis of their modes of action. Representatives of these were individually added to the buffered L-dopa solution used in the concentrations detailed below, and sections were incubated as before for 24 h at 37° C:

General enzyme inhibitors. Hydrogen sulphide (saturated solution), sulphur dioxide (saturated solution), potassium cyanide (1% and 0·1%).

Cytochrome oxidase inhibitors. Sodium azide (0·001 M), phenylurethrane (0·003 M).

Substances which prevent the oxidation of tyrosine to dopa. 'Tween 20' (10% solution), hydroquinone (0·07 M).

Copper-binding tyrosinase inhibitors. Sodium diethyldithiocarbamate (0·01 M), thiourea (1 M), phenylthiourea (0·01 M), α-naphthylthiourea (0·01 M).

Competitive inhibitor of tyrosinase. 4-chlororesorcinol (0·01 M).

Results

Monophenolase activity in nigra cells

The method used to demonstrate monophenolase activity resulted in a negative response. No blackening of the cytoplasm was detectable in the nigra cells of the animals employed after incubation in L-tyrosine.

Diphenolase activity in nigra cells

The method used to demonstrate diphenolase activity resulted in a positive response, consisting of intense blackening of the nigra cells in both animals (fig. 1, A). No blackening occurred in the nigra cells in sections incubated in buffer solution alone (fig. 1, B). The blackening of nigra cells was due to the presence of varying concentrations of small black granules of pigment, produced by the conversion of dopa to melanin. Small numbers of isolated pigmen granules were also scattered uniformly throughout the sections, but pigment was most heavily concentrated specifically in the nigra cells. A numbe of isolated cells in the reticular formation of the mid-brain were also blackened whereas the cells of other nuclear groups were almost indistinguishable fron the surrounding background (fig. 1, C). In the less intensely blackened nigr cells, the pigment granules were separate (fig. 1, D), while in heavily blackene cells they were concentrated into a dense, black mass (fig. 1, E). Pigmen granules were confined to the cytoplasm of nigra cells and were not found i the nucleus, nor did they extend into the cell processes (fig. 1, F). In some cell granules were concentrated around the nucleus, producing a darkly stainin 'perinuclear ring' (fig. 1, G). Granules were also concentrated in the tissue i the immediate vicinity of the nigra cells.

Variations in physical factors influenced the intensity of the reaction. Incubation at a pH below 6·8 produced no blackening of nigra cells, but a pH 6·8 to 7·4 the black cells stood out distinctly from the pale background The intensity of the reaction was not greatly increased by incubation at a pH above 7·4, but the scattered background pigmentation was markedly intensified. Incubation of sections in different concentrations of L-dopa showed tha the higher the concentration of substrate employed, the greater was the intensity of blackening of nigra cells. Within limits, the longer the period of incubation the greater was the intensity of the result, the maximum blackenin of nigra cells occurring after 24 h incubation. Even after 2 h a few cells ha blackened selectively in the substantia nigra. The maximum intensity of the blackening of nigra cells occurred when sections were incubated at 37° C No reaction took place when sections were incubated at 4° C, and sections incubated at 18° C took up to 72 h to exhibit blackening of nigra cells; after incubation at 60° C the whole section darkened, but there was no specific blackening of nigra cells.

Fig. 1 (plate). A, the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa.

B, the nucleus substantiae nigrae of the adult monkey after incubation in buffer solution alone.

C, a cell from the nucleus ruber of the adult monkey after incubation in L-dopa. The cytoplasm is pale and free of pigment.

D, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. The cytoplasm of this lightly blackened cell is packed with black pigment granules.

E, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. The cytoplasm of this heavily blackened cell is completely obscured by a dense mass of black pigment.

F, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. Pigment is concentrated within the cytoplasm of the cell, and granules lie in the surrounding tissue. The cell nucleus and nerve processes are free of pigment.

G, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. Pigment is concentrated in a 'perinuclear ring'.

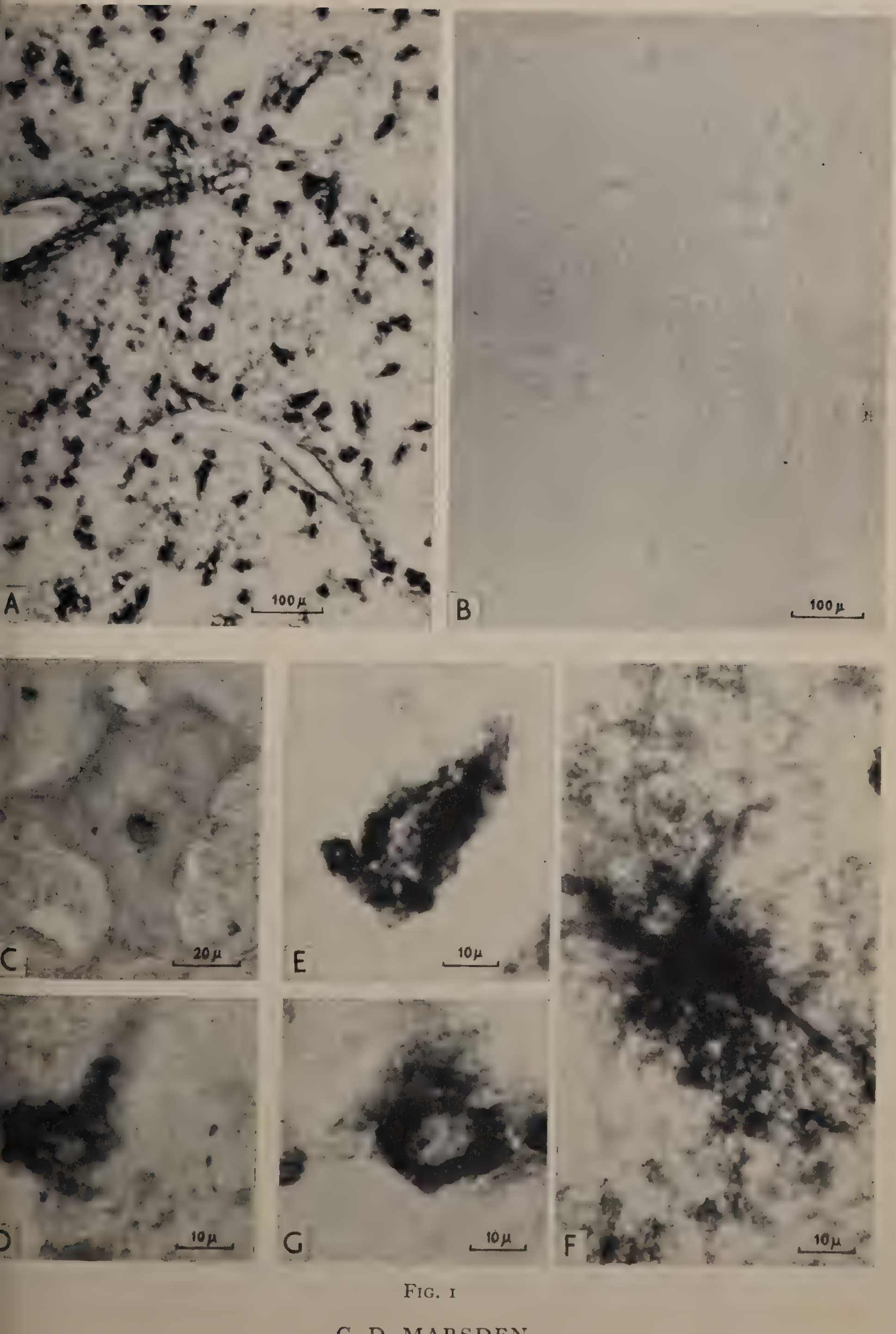

Fig. 1

C. D. MARSDEN

Of the inhibitors employed, the majority, namely hydrogen sulphide, sulhur dioxide, potassium cyanide, sodium diethyldithiocarbamate, thiourea, henylthiourea, α-naphthylthiourea, and 4-chlororesorcinol, prevented the onversion of dopa to melanin and the subsequent blackening of nigra cells. ections incubated in L-dopa, with the addition of these inhibitors individully, showed no differences from similar sections incubated in buffer solutions lone. Blackening of nigra cells still occurred when sections were incubated in he presence of sodium azide, phenylurethrane, 'tween 20', and hydroquinone.

DISCUSSION

The negative results obtained with the reaction for the monophenolase ctivity of tyrosinase could be interpreted as indicating the absence of this nzyme in adult nigra cells. However, the positive result obtained with the eaction for the diphenolase activity of tyrosinase excludes this possibility. he specificity of this latter reaction for diphenolase activity has been conrmed by studying the effects of variations of physical factors and by the use f inhibitors of tyrosinase.

Although the oxidation of dopa to melanin can occur in the absence of an enyme in solutions above pH 7·0 (Lerner, Fitzpatrick, Calkins, and Summerson, 949), a positive diphenolase reaction in nigra cells was obtained at pH 6·8. he cytochrome oxidase group of enzymes can also oxidize dopa to melanin Herrman and Boss, 1945), but the addition of two inhibitors of cytochrome xidase activity, sodium azide and phenylurethrane (Pearse, 1953), did not nterfere with the positive diphenolase reaction in nigra cells. Finally, the iphenolase reaction was completely suppressed by specific inhibitors of tyronase. The copper-binding inhibitors employed acted by forming weakly issociable complexes with the copper moiety of the enzyme (Lerner and Fitzatrick, 1950), the presence of copper ions being essential for tyrosinase action erner, Fitzpatrick, Calkins, and Summerson, 1950), while the competitive hibitor acted as a competitive substrate for the enzyme (Fitzpatrick and ukita, 1959).

The absence of monophenolase activity in the presence of undoubted dihenolase activity in adult nigra cells is puzzling. In this respect these pigented cells resemble certain pigmented melanocytes in the skin and other ssues. Monophenolase activity cannot be detected in skin melanocytes unless gment formation is occurring, nor in the melanocytes of human grey hair, en though these cells retain the ability to oxidize dopa (Fitzpatrick, Brunet, d Kukita, 1958). It is probable that monophenolase and diphenolase activity n be detected together only in melanocytes which are in the process of active gment formation. The failure to detect monophenolase activity in adult gra cells may indicate that pigment formation has temporarily or peranently ceased. However, the demonstration of diphenolase activity in the gra cells of two animals that possess pigmented cells in the nucleus subantiae nigrae suggests that tyrosinase plays an important role in the formaon of the pigment, which is probably melanin derived from tyrosine.

My thanks are due to Professor D. V. Davies for his encouragement an help in this work; to Dr. W. Hewitt for his criticism of the manuscript; t Mr. D. N. Petts for technical assistance; and to Mr. J. S. Fenton for th photomicrographs.

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Some Modifications of Holmes's Silver Method for Insect Central Nervous Systems

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With two plates (figs. 1 and 2)

SUMMARY

Two main modifications of Holmes's method are described. In both the amount of silver nitrate in the impregnating bath is increased, and larger quantities of higher pyridine derivatives are substituted for pyridine. The preferred method for general purposes employs 2·4% 2:6 lutidine at pH 8·4, and gives a bluish picture with improved selectivity of fibre staining. The use of 1·6% 2:4:6 collidine gives a reddish picture, with highly selective fibre staining, and sharp resolution of neurosecretory cells. Minor modifications of pre-treatment and reduction procedures are also described.

INTRODUCTION

HOLMES'S method for the silver impregnation of paraffin sections was originally devised for the vertebrate peripheral nervous system (Holmes, 1947). It has since been used on invertebrate material with varying degrees of success (Vowles, 1955; Larsen, 1960). Certain materials, e.g. the brains of lepidopterous larvae (Van der Kloot and Williams, 1954) have proved difficult to stain.

Unsatisfactory impregnation of the brains of adult tussore silk moths (*Antherea mylitta*) and of *Automeris* spp. has led to an exploration of some modifications of Holmes's method other than mere manipulation of the pH of the impregnating solution.

MATERIALS AND METHODS

Brains of the desert locust (*Schistocerca*), the honey bee (*Apis*), *Antherea*, and *Automeris* spp. were fixed for 24 h in alcoholic Bouin's solution, washed in 50% alcohol, and embedded in 56° C wax after dehydration through alcohols, cedarwood oil, and benzene. Sections were cut at 10 μ. After dewaxing, the sections were brought to distilled water as rapidly as possible. They were then processed as follows:

1. 20% silver nitrate.	2 or 3 h
2. Rinse in distilled water.	5 min

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3. Incubate in the following solution at 37° C:

M/5 boric acid	27·5 ml	
M/20 borax	22·5 ml	
1% silver nitrate solution	5 to 10 ml	
pyridine or derivative	2 to 6 ml	
distilled water	250 ml	16 to 20 h
(pH 8·4 at 20° C, by B.D.H. narrow-range indicator strips)		

4. Place without washing in the following solution:

hydroquinone	3 g	
sodium sulphite	30 g	
distilled water	300 ml	3 min

The initial temperature of this reducing bath was 60° C. It fell to 55° C at the end of the treatment.

5. Wash in running tap-water. 3 min
6. Rinse in distilled water. 3 min
7. Tone in 0·2% sodium gold chloride solution. variable (1 to 3 min)
8. Rinse in distilled water. 1 min
9. Reduce in 2% oxalic acid. 5 min
10. Wash in distilled water. 3 min
11. Fix in 5% sodium thiosulphate. 1 min
12. Wash thoroughly in distilled water, dehydrate, and mount.

The following substances have been used in the impregnating bath:

pyridine.	6 ml	with 10 ml silver nitrate in 250 ml of buffer
α-picoline (1-methylpyridine)	6 ml	
2:4 / 2:5 lutidine (dimethylpyridine)	6 ml	
2:6 lutidine (dimethylpyridine)	6 ml	
2:4:6 collidine (trimethylpyridine)	4 ml	
2-methyl-5-ethyl-pyridine	2 ml	with 5 ml silver nitrate in 250 ml of buffer

Solutions containing 2:4 / 2:5 lutidine were filtered before use. In all cases thorough shaking was necessary during the addition of the usually poorly miscible higher derivatives. The amounts of collidine and of 2-methyl-5-ethyl-pyridine are about the maximum which is miscible without separating out during incubation.

A few tests were made with cat and rat brains perfused with formalin solution and cut at 15 μ. For these materials the amount of silver in the impregnating baths was reduced to 3 ml of 1% silver nitrate.

FIG. 1 (plate). A, α-lobe of corpus pedunculatum of *Apis*, cut transversely towards the distal end. 2:6 lutidine method.

B, α-lobe of *Apis*, cut at the same level as A. Pyridine method.

C, transverse section of the medial part of the protocerebrum of *Automeris aurantiaca*. 2:6 lutidine method.

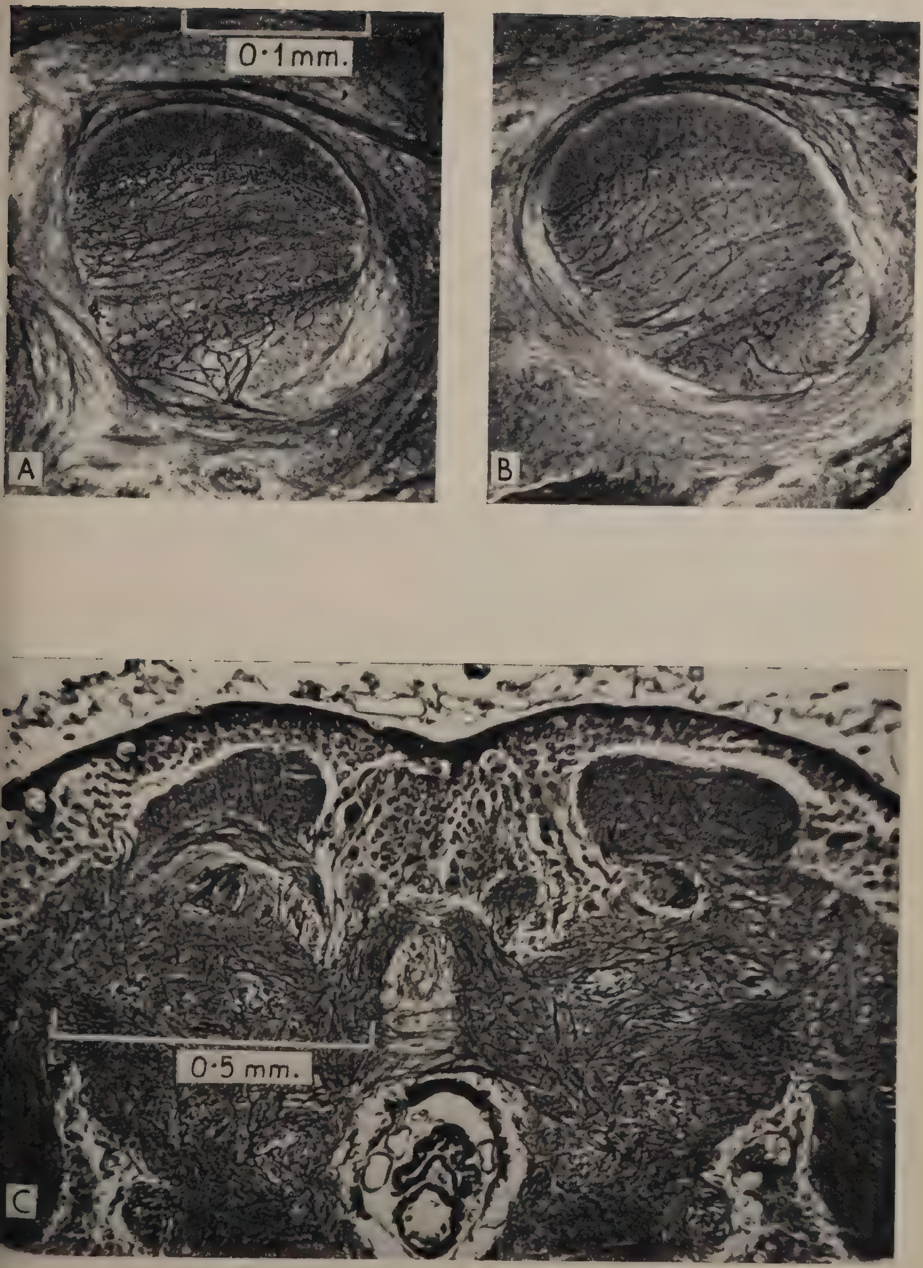

FIG. 1

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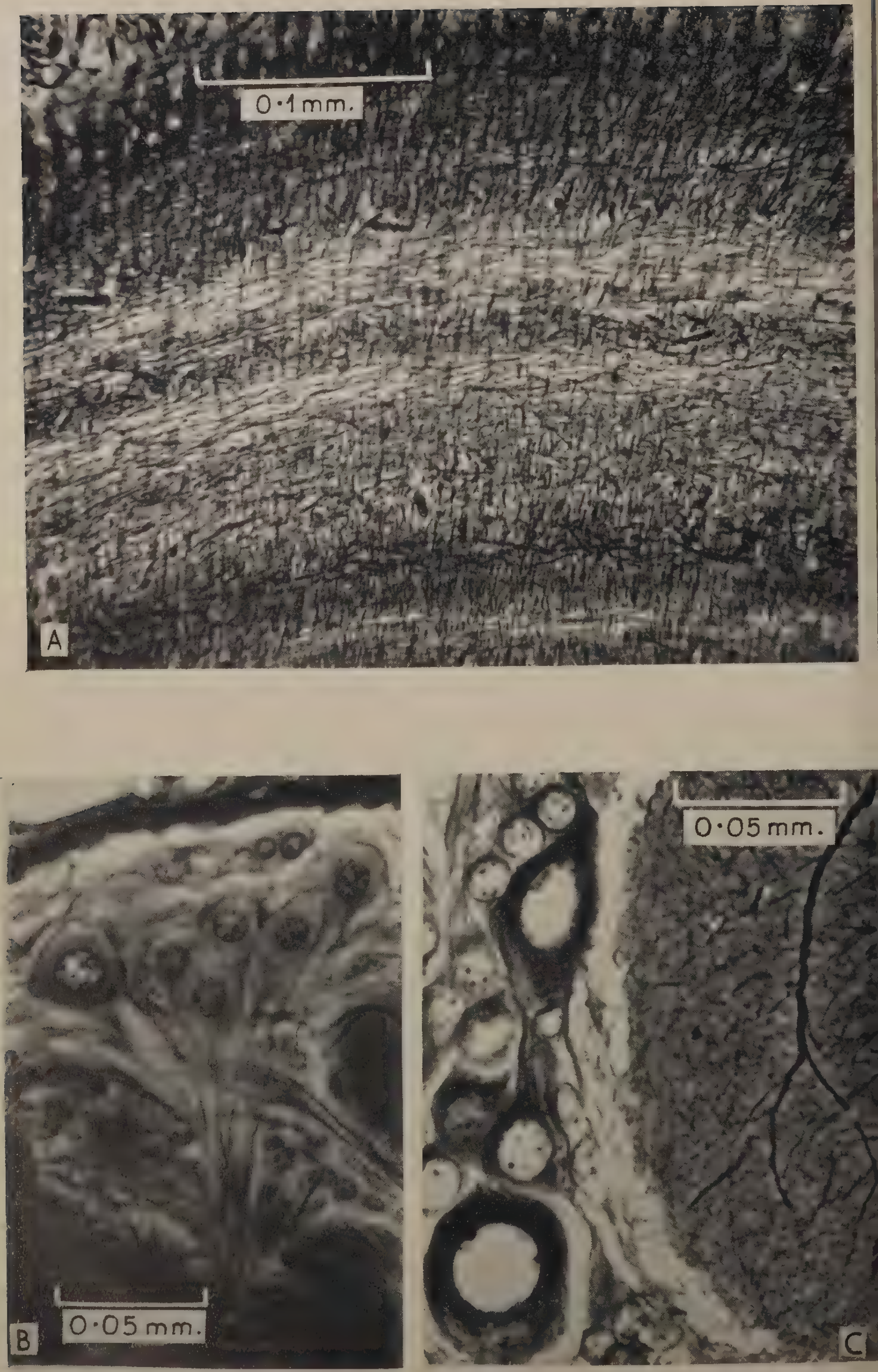

FIG. 2

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Critical comparisons were confirmed with sections derived from single brains.

Results

Some of the results obtained are shown in figs. 1 and 2.

Differences between materials. Good impregnation of the locust and bee brains was obtained with all the derivatives tested. Results with *Antherea* and *Automeris* were always adequate and sometimes excellent.

Differences between the pyridine derivatives. Despite considerable variation between batches, the following differences were clear-cut and replicable. Increasing the number of alkyl groups on the pyridine molecule leads to an ncrease in the specificity of fibre staining, which does not appear to be the esult of increased silver uptake, but due to a reduction of the degree of background staining of the neuropile. There is a consistent impression that the proportion of small fibres separately stained is greatest in the lutidine preparaions. There is also an increase in the differential staining of the fibre tracts. Pyridine preparations show, for example, some differences in the staining reactions of the large tracts linking the optic centres, and the fine fibres of the calyces of the mushroom bodies. These differences are exaggerated in the utidine and collidine preparations and also in some *Automeris* brains processed with 2-methyl-5-ethyl-pyridine. Further, there is a decrease in the taining of the normal nerve-cell bodies supplying the neuropile. In lutidine preparations the nuclei are well stained, while the cytoplasm is clear. In collidine preparations the nuclei, too, are more weakly stained. Beyond this, there s an increase in the differential staining of the neurosecretory cells both in the medial and medio-lateral groups of the protocerebrum, and in the suboesophageal region. This increase does not extend to the neurosecretory cell nuclei, nd neurosecretory cells do not invariably show denser staining than that of he neurones surrounding them; the latter variable may perhaps prove to relate to secretory phase.

In addition there are differences in the general colour of sections stained in he presence of the various derivatives. The usual colours assumed by fibres of medium thickness of the protocerebral neuropiles of both *Schistocerca* and *Antherea* are:

pyridine—reddish purple
α-picoline—reddish-bluish
lutidines—very bluish
collidine—reddish
2-methyl-5-ethyl pyridine—reddish-purple (*Automeris* only)

The few results obtained with vertebrate tissues showed similar trends.

FIG. 2 (plate). A, second optic glomerulus of *Schistocerca*. 2:6 lutidine method.

B, group of 6 medio-lateral protocerebral neurosecretory cells from *Antherea*. 2:6 lutidine method.

C, portion of a calyx of corpus pedunculatum of *Schistocerca* with a large branching afferent bre (on right), and adjacent secretory and non-secretory cell-bodies (on left). 2:4:6 collidine method.

DISCUSSION

The mechanisms of silver impregnation are still imperfectly understood (Peters, 1955; Wolman, 1955) and these modifications to Holmes's method must be regarded as empirical. The results obtained with the various pyridine derivatives are sufficiently striking to suggest that they deserve to be tried as possible alternatives to pyridine in all those silver-staining methods which involve treatment or pre-treatment with solutions containing pyridine.

The following general conclusions may be reached. α-Picoline shows no advantages over pyridine that do not appear in more marked form in lutidine preparations. The blueness of the lutidine preparations is a definite advantage, and recommends its routine use. No distinction could be made between 2:6 lutidine and the 2:4 / 2:5 lutidine mixture; both yielded equally good preparations, which were invariably superior to pyridine preparations from the same batch; but since none of the samples of 2:4 / 2:5 lutidine used was completely miscible with water, the use of wholly miscible 2:6 lutidine is to be preferred. This modification is now used as a standard procedure for the examination of *Antherea* and *Automeris* brains after surgical interference in early pupal development, and it has yielded uniformly satisfactory results.

The results obtained with solutions containing collidine suggest that this substance may well be of value where a sharp discrimination of neurosecretory cells is required, and for fine detail. Sections treated with 2-methyl-5-ethyl pyridine, however, have tended to show a poor definition of fine fibres in the neuropile, and a coarsely granular impregnation.

The general trends observed with insect material were also noted in the few preparations which were made of vertebrate material. Here the decrease in the staining and resolution of background structure which follows the use of higher pyridine derivatives is a disadvantage. While it has proved that lutidine and collidine can be usefully employed for special purposes, e.g. in modifications of the Nauta-Gygax technique for degenerating fibres (Guillery, Shirra, and Webster, 1961), most probably the benefits which it confers over pyridine for vertebrate brains are no more than marginal.

Although the methods described have invariably given satisfactory results with brains of *Schistocerca*, *Apis*, *Antherea*, and *Automeris*, a few attempts to stain sphingid brains were not successful. As with the pyridine-silver technique, particular materials will doubtless demand appropriate alterations to the pH and silver concentration of the impregnating bath, and the selection of an optimal fixative solution.

I am grateful to Dr. R. W. Guillery for supplying sections of cat and rat brains perfused with formalin solution, and for commenting on the results obtained from them, and to Roche Products Ltd. for a sample of 2-methyl-5-ethyl pyridine. This work was started under a grant from the University of London Central Research Fund, and continued with support from the United States Public Health Service (Project RG-7109).

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a, aster; *ab*, acroblast; *af*, axial filament; *as*, acrosome; *b*, basal granule (blepharoplast); *bb*, brush border; *bm*, basement membrane; *c*, centriole; *ch*, chromosome; *cl*, cilium; *cm*, cell membrane; *ctm*, centromere (kinetochore); *cts*, centrosphere (idiozome); *er*, endoplasmic reticulum (ergastoplasm); *f*, flagellum; *g*, γ-cytomembrane ('Golgi' membrane); *l*, lipid droplet; *m*, mitochondrion; *n*, nucleus; *nl*, nucleolus; *nm*, nuclear membrane; *s*, spindle; *v*, vacuole.

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